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14. ABSTRACT

We have made good progress during the current funding period. We have established systems to generate human and mouse B7-H3 fusion proteins and then used them to immunize B7-H3 gene knock mice. Two of immunized B7-H3-/- mice generated good anti-B7-H3 antibodies in the blood and therefore one of these mice were used to generate hybridomas. We also have developed FACS-based approach to screen hybridomas and to exclude cross-reactive hybridomas. Finally, we have generated eight B7-H3-specific mAbs.

15. SUBJECT TERMS
B7x; B7-H3; T cell coinhibition; prostate cancer; monoclonal antibodies; immunotherapy.

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Table of Contents

Page

1. INTRODUCTION

T cells of the immune system are the major combatants against cancers. T cell activation, proliferation, differentiation to effector function and memory generation are determined by both positive costimulation and negative coinhibition, generated mainly by the interaction between the B7 family and their receptor CD28 family. We have discovered the newer members of T cell costimulatory/coinhibitory B7 family, such as B7x and B7-H3, and found that both B7x and B7-H3 down-regulates immune responses via negative T cell costimulation (coinhibition). Therefore, we are developing a new concept: T cell coinhibition. Our central hypothesis of this grant is that blockade of B7x and B7-H3 generates therapeutic tumor immunity against prostate cancer.

We finished the largest investigation of B7 family molecules in human malignancy, 823 prostatectomy patients for whom a minimum of 7 year follow-up data was available. This study reveals that prostate cancer patients with strong expression of B7x or its closest homologue B7-H3 by tumor cells are significantly more likely to have disease spread at time of surgery, and are at significantly increased risk of clinical cancer recurrence and cancer-specific death. In addition, we also find that B7x and B7-H3 are over-expressed in human ovarian carcinomas and breast cancers. These basic studies and clinical observations have formed the foundation that targeting B7x and/or B7-H3 could be developed as novel immunotherapies against human prostate cancer. The first aim of this proposal is, therefore, to develop new immunotherapeutic strategies against prostate cancer by anti-B7x and ant-B7-H3. The second aim of this proposal is, therefore, to examine combination therapy of anti-B7x/anti-B7-H3 with other therapies.The understanding of these two novel pathway is anticipated to provide new targets for therapeutic interventions that will aid the growing numbers of prostate cancer patients. In addition, it is expected that the proposed research will fundamentally advance the fields of T cell coinhibition in cancer.

2. KEYWORDS

B7x; B7-H3; T cell coinhibition; prostate cancer; monoclonal antibodies; immunotherapy.

3. ACCOMPLISHMENTS

What were the major goals of the project?

Task 1. Develop new immunotherapeutic strategies against prostate cancer by anti-B7x and ant-B7-H3 (Months 1-36).

1a.Generation of mAbs to human and mouse B7-H3 (Months 1-12).

1b.Effectiveness of B7x and/or B7-H3 blockade in a subcutaneous prostate tumor model (Months 6-24).

1c.Effectiveness of B7x and/or B7-H3 blockade in treatment of prostate tumor metastasis (Months 12-30).

1d.Effectiveness of B7x and/or B7-H3 blockade in treatment of primary prostate tumor (Months 12-36).

1e.Effect of B7x- or B7-H3-specific mAbs on T cell function in vivo in humanized NSG mice (Months 12-24).

Task 2. Examine combination therapy of anti-B7x/anti-B7-H3 with other therapies (Months 12-36).

2a.Synergy between anti-B7x/B7-H3 therapy and blockade of PD-1 or CTLA-4 (Months 18-30).

2b.Synergy between B7x/B7-H3 blockade and regulatory T cell depleting (Months 18-30).

2c.Potential mechanism of anti-B7x/B7-H3 therapy: blockade of B7-mediated T cell immunosuppression (Months 12-36).

2d.Potential mechanism of anti-B7x/B7-H3 therapy: effect on the generation of induced regulatory T cells (iTreg) and myeloid-derived suppressor cells (MDSCs) (Months 12-36).

2e.Potential mechanism of anti-B7x/B7-H3 therapy: antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity (Months 12-30).

What was accomplished under these goals?

Task 1. Develop new immunotherapeutic strategies against prostate cancer by anti-B7x and ant-B7-H3 (Months 1-36).

- We have B7-H3 gene knock-out mice. Therefore we took advantage of these mice for generating mAbs against both human and mouse B7-H3. Briefly, B7-H3 knock-out mice were immunized with 100ug of human B7-H3-Ig fusion protein, after 3 weeks the mice were immunized with 100 ug of mouse B7-H3-Ig fusion protein with hopes to produce cross-reactive antibodies. Two immunized mice generated good anti-B7-H3 antibodies in sera (Fig. 1), suggesting our immunization protocol worked well.

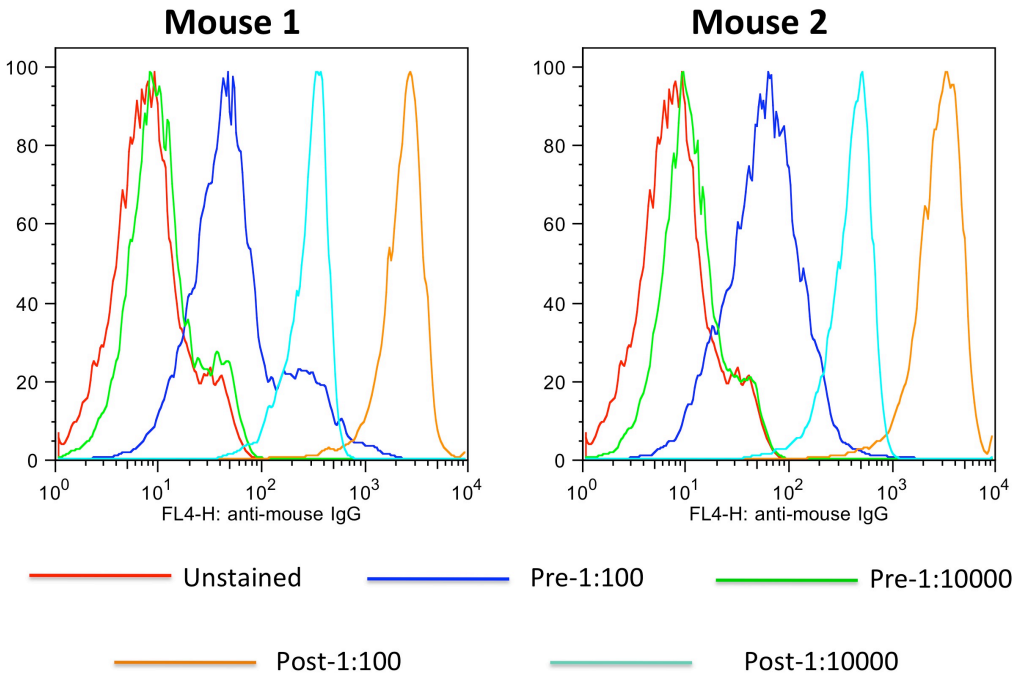

Figure 1. Immunization and screen of anti-B7-H3 positive mice. B7-H3-/- mice were immunized with human and mouse B7-H3-Ig fusion proteins. After immunization, two mice generated good anti-B7-H3 antibodies in their blood.

- One of above two mice was finally boosted with 100 ug of human B7-H3-Ig. Four days after the final boost half of the spleen was harvested and fused to a myeloma cell line and plated over 22 plates in preparation for a primary screening.
- ELISA is the traditional method that is used to screen positive wells for individual clones, but our lab has recently developed a FACS based protocol to perform the screening that is much faster and more suitable for our purposes. For example we would use 3T3 cells expressing hB7H3 tagged to YFP and 3T3 cells expressing another protein that is YFP negative. We added these cells in a 50/50 ratio to an individual well than added supernatant from one of the many potential clones to the well for 30 minutes which we hoped to contain antibodies against B7-H3. Then we added a conjugated anti-mouse Ig for 30 minutes and went to FACS to look for positive hits (Fig 2.)

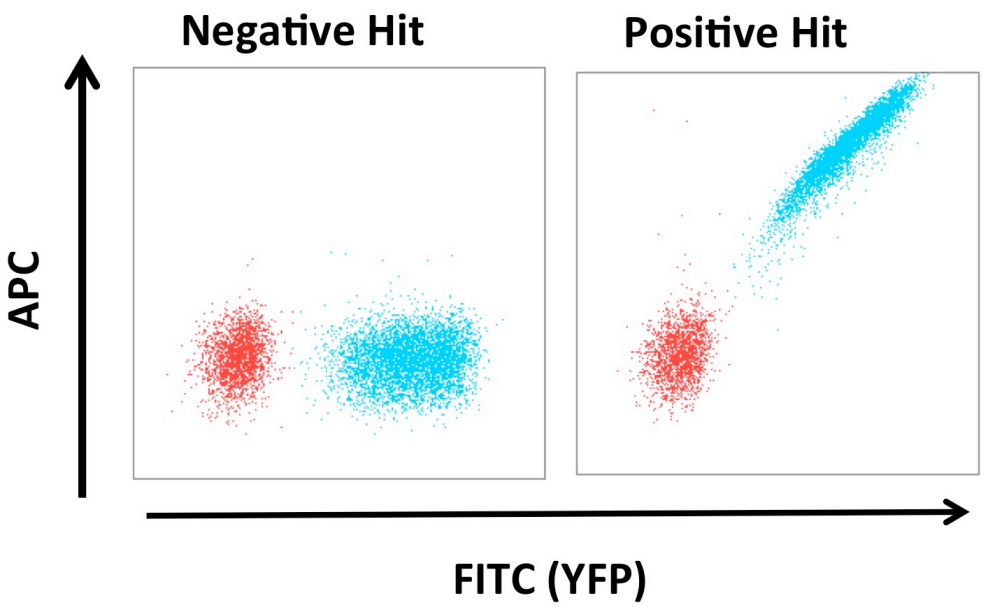

Figure 2. Generation and screen of monoclonal antibodies against human and mouse B7-H3. A representative example of two possible outcomes of the screen. The first are the two different populations of 3T3 cells on the x-axis with none of them bound to antibody, and the second graph showing the YFP positive population binding human B7H3 antibody.

- We went through and screened all plates and sub-cloned each positive hybridoma 3 times to ensure a monoclonal population. We were able to obtain a total of 9 different clones.
- Following the subcloning of these hybridomas the next step was to check and make sure that these antibodies were specific for only B7-H3 and did not cross-react with other B7 ligand and CD28 family members. In our lab we have generated 3T3 cells to express all of the B7 ligand and CD28 receptor family members and tested all clones to exclude ones cross-reactive to other B7/CD28 members (Fig. 3).
- 8E7 and 13A9 are two representative clones we have, which showed good binding to human B7H3 (left) and no binding to any of the other B7 and CD28 family members (right) (Fig. 3). On the other hand, one of the 9 clones 12E11, in addition to binding highly B7H3 it also bound to all of the other family members (Fig. 3), making it non-specific and unuseable for our purposes. So this left us with 8 hybridomas to work with.

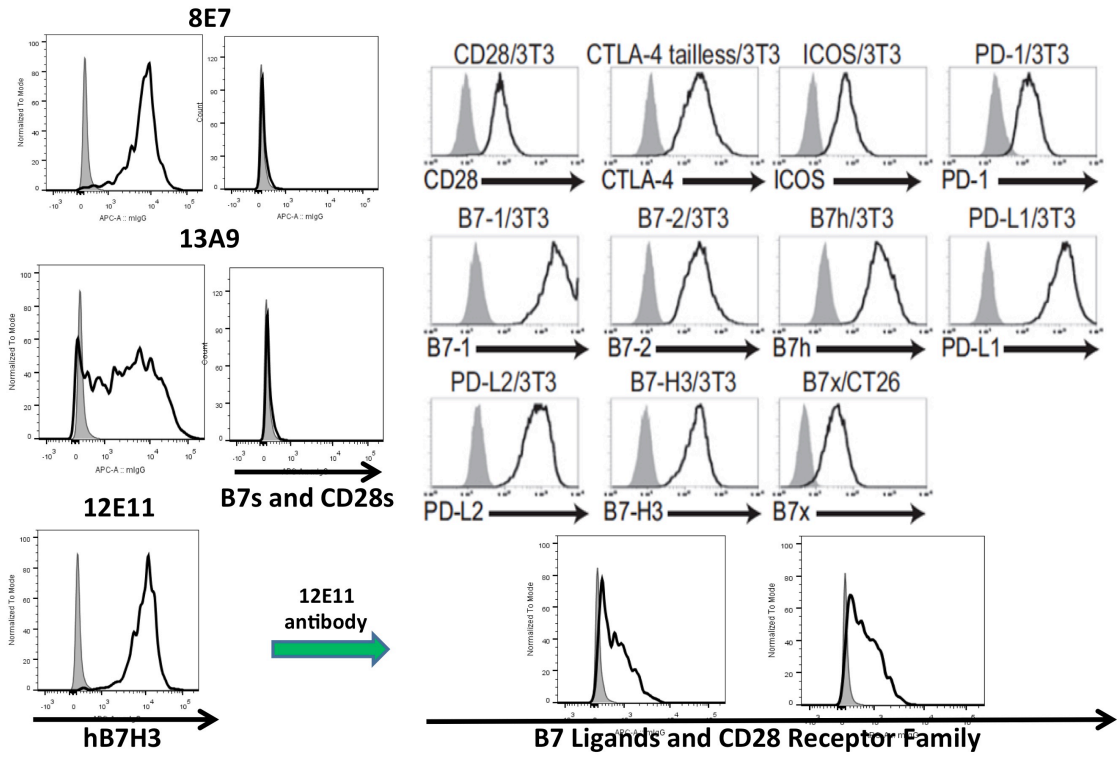

Figure 3. Specificity of our nine clones against human B7-H3.

What opportunities for training and professional development has the project provided?

Nothing to Report

How were the results disseminated to communities of interest?

Nothing to Report

What do you plan to do during the next reporting period to accomplish the goals?

We will 1) screen our above mAbs against B7-H3 to find which ones are able to generate anti-tumor immunity; and 2) Generate new mAbs against human B7x and test their ability to induce anti-tumor immunity.

4. IMPACT

Nothing to Report

5. CHANGES/PROBLEMS

None

6. PRODUCTS (Publications)

1) Zhao H, Bauzon F, Bi E, Yu JJ, Fu H, Lu Z, Cui J, Jeon H, **Zang X**, Ye BH, Zhu L (2015). Substituting threonine^{187} with alanine in p27Kip1 prevents pituitary tumorigenesis by two-hit loss Of *Rb1* and enhances humoral immunity in old age. **Journal of Biological Chemistry**, 290:5797-5809
PMCID: PMC4342489

2) Janakiram M, Chinai JM, Zhao A, Sparano JA, **Zang X** (2015). HHLA2 and TMIGD2: New immunotherapeutic targets of the B7 and CD28 families. **OncoImmunology**, 4: e1026534-1- e1026534-3
PMCID: PMC4570140

3) Chinai JM, Janakiram M, Chen F, Chen W, Kaplan M, **Zang X** (2015). New Immunotherapies targeting the PD-1 pathway. **Trends in Pharmacological Sciences**, 36:587-595.
PMCID: PMC4562806

4) Assal A, Kaner J, Pendurti G, **Zang X** (2015). Emerging targets in cancer immunotherapy: beyond CTLA-4 and PD-1. **Immunotherapy**,
PMID: 26567614 [PubMed - in process]

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	Xingxing Zang	Kim Ohaegbulam	Elodie Picarda	Qizhe Sun	Xiaoshen Dong
Project Role:	PI	MD-PhD student	Postdoc fellow	Visiting scholar	Visiting scholar
Nearest person month worked:	2	6	4	4	4
Contribution to Project:	Oversaw the project	Immunized B7-H3-/- mice; hybridoma fusion and screen	Developed protocols for B7-H3 staining	Made vectors to express B7-H3-Ig proteins	Purified B7-H3-Ig proteins
Funding Support:	NIH, DOD	NIH, DOD	NIH DOD	Fellowship	Fellowship

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
Nothing to Report

What other organizations were involved as partners?
Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

None

9. APPENDICES

Next page

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Substituting Threonine 187 with Alanine in p27Kip1 Prevents Pituitary Tumorigenesis by Two-Hit Loss of *Rb1* and Enhances Humoral Immunity in Old Age*

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Background: p27T187A knockin mice facilitate studying p27Kip1 protein in physiology.
Results: p27T187A knockin prevented pituitary tumorigenesis in $Rb1^{+/-}$ mice and enhanced humoral response to immunization in older age.
Conclusion: Phosphorylation of p27T187 is important in *Rb1*-deficient tumorigenesis and immunity in aging.
Significance: Specific cancer is identified for treatment by inhibiting Skp2/Cks1-p27T187p interaction and new directions are revealed in understanding immunity decline in elderly.

p27Kip1 (p27) is an inhibitor of cyclin-dependent kinases. Inhibiting p27 protein degradation is an actively developing cancer therapy strategy. One focus has been to identify small molecule inhibitors to block recruitment of Thr-187-phosphorylated p27 (p27T187p) to $SCF^{Skp2/Cks1}$ ubiquitin ligase. Since phosphorylation of Thr-187 is required for this recruitment, p27T187A knockin (KI) mice were generated to determine the effects of systemically blocking interaction between p27 and Skp2/Cks1 on tumor susceptibility and other proliferation related mouse physiology. $Rb1^{+/-}$ mice develop pituitary tumors with full penetrance and the tumors are invariably $Rb1^{-/-}$, modeling tumorigenesis by two-hit loss of *RB1* in humans. Immunization induced humoral immunity depends on rapid B cell proliferation and clonal selection in germinal centers (GCs) and declines with age in mice and humans. Here, we show that p27T187A KI prevented pituitary tumorigenesis in $Rb1^{+/-}$ mice and corrected decline in humoral immunity in older mice following immunization with sheep red blood cells (SRBC). These findings reveal physiological contexts that depend on p27 ubiquitination by $SCF^{Skp2\text{-}Cks1}$ ubiquitin ligase and therefore help forecast clinical potentials of Skp2/Cks1-p27T187p interaction inhibitors. We further show that GC B cells and T cells use different mechanisms to regulate their p27 protein levels, and propose a T helper cell exhaustion model resembling that of stem cell exhaustion to understand decline in T cell-dependent humoral immunity in older age.

Cyclin-dependent kinase (Cdk)[2] inhibitor p27Kip1 (p27) binds cyclin/Cdk and inhibits their kinase activity. Since cyclin/Cdk drives the cell cycle engine, p27 and its family members p21 and p57 are best known as negative regulators of cell proliferation. p27 knock-out (KO) mice are larger and heavier by about 20% over wild type (WT) mice and develop pituitary intermediate lobe (IL) tumors, providing *in vivo* evidence for the anti-proliferative functions of p27 (1–3). p27 ck-mice [RxL32 to AxA32 knockin (KI) to disrupt p27 binding to cyclins, and FDF64 to ADA64 KI to disrupt p27 binding to Cdks] phenocopied p27 KO mice in larger body size and pituitary tumorigenesis, confirming the biochemical mechanisms of p27 function *in vivo* (4).

The best known mechanism for regulating p27 expression is its polyubiquitination leading to degradation in the proteasome, and the best known regulator of p27 ubiquitination is Skp2, which is the substrate recruiting subunit of the SCF^{Skp2} ubiquitin ligase (5). SCF^{Skp2} has a growing list of substrates. For recruiting p27, threonine 187 of p27 (p27T187) must be phosphorylated (6, 7) and an accessory protein, Cks1, must be present (8, 9). The phosphorylated threonine 187 fits into a pocket formed by Skp2 and Cks1 to enable stable interaction between p27 and Skp2/Cks1 (10); p27 is therefore ubiquitinated in the $SCF^{Skp2/Cks1}$-p27T187p complex.

p27T187A mutation (substitution of threonine with alanine) renders p27T187 unphosphorylable and, therefore, cannot be ubiquitinated by $SCF^{Skp2/Cks1}$. To test the biological significance of ubiquitination of p27T187p by $SCF^{Skp2/Cks1}$, p27T187A KI mice were generated (11).

Studies of cultured $p27^{T187A/T187A}$ mouse embryonic fibroblasts (MEFs) in serum starvation (to maintain MEFs in G0) and re-stimulation (to stimulate MEFs to proliferate) revealed re-

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[2] The abbreviations used are: Cdk, cyclin-dependent kinase; GC, germinal center; SRBC, sheep red blood cell; KI, knockin; KO, knock-out; MEF, mouse embryonic fibroblast; FACS, fluorescence-activated cell sorting; PNA, peanut agglutinin; IHC, immunohistochemistry; IF, immunofluorescence.

accumulation of p27 protein when cells entered S phase to levels seen in G0 phase, demonstrating that ubiquitination of p27T187p by $SCF^{Skp2/Cks1}$ is responsible for p27 protein degradation in S-G2 phases of the cell cycle (11). The biological effects of p27T187A KI varied with cell types. In MEFs stimulated by serum refeeding, p27T187A KI reduced S phase cell fraction by 20%. When splenic CD4+ T cells were activated by anti-TCR (T cell receptor), S phase cell reduction reached 80% (11). We will discuss the latter result further below.

At organismal level, since cells in adult tissues are mostly in quiescence, no abnormal p27 protein accumulation was detected in various tissues in $p27^{T187A/T187A}$ mice (11). $p27^{T187A/T187A}$ mice provide a gain-of-protein stability tool to study the effects of p27 protein accumulation in S-G2 of proliferating cells in physiological settings. For examples, Malek *et al.* (11) reported that healing of circular skin punch wounds was delayed by about 2-fold in $p27^{T187A/T187A}$ mice compared with WT mice when sizes of wounds were measured at 4.5 days after wounding. Proliferation of dermal keratinocytes around the wounds was reduced by 2.5 fold as measured by BrdU labeling. However, $p27^{T187A/T187A}$ mice grew larger than WT mice by about 20% in body weight at 80 days of age. Thus, p27T187A mutation produced proliferation-inhibitory as well as proliferation-stimulatory phenotypes. Mechanisms underlying the large body size phenotype of $p27^{T187A/T187A}$ mice remains to be determined.

Later studies examined $p27^{T187A/T187A}$ mice in other physiological processes involving cell proliferation, such as liver regeneration after partial hepatectomy (12), atherosclerosis and atheroma formation in ApoE KO mice on fat feeding (13), lung tumorigenesis following spontaneous activation of endogenous *Kras* (14), and multi-organ tumorigenesis following administration of carcinogen ENU to 15-day-old mice (14). Interestingly, in none of these experimental systems was p27T187A KI found to alter the main pathological/physiological outcomes. Only the ratios of histopathologically diagnosed carcinomas over adenomas were reduced in intestines of ENU-treated $p27^{T187A/T187A}$ mice compared with WT mice at necropsy (14).

At the same time, inhibitors of the Skp2/Cks1-p27T187p interaction are being actively developed as therapeutics for cancer (15–17) with the rationale that inhibiting this interaction would specifically stabilize p27 protein without affecting other substrates of SCF^{Skp2}, thereby minimizing side effects. $p27^{T187A/T187A}$ mice could model inhibitor treatment to block Skp2/Cks1-p27T187p interaction. Altogether, it is highly desirable and timely to define the type of cancers and normal physiological processes affected in $p27^{T187A/T187A}$ mice.

In this study, we examined the role of p27T187A KI in two experimental models. In the first, we crossed $p27^{T187A/T187A}$ mice with $Rb1^{+/-}$ mice to determine the effects of p27T187A KI on pituitary tumorigenesis in $Rb1^{+/-}$ mice, which models two hit loss of *RB1* in humans and is fully penetrant. Next, we tested $p27^{T187A/T187A}$ mice for T cell-dependent immunization response, which depends on B cell clonal expansion, diversification, and affinity selection within the germinal centers (GCs, (18)) in secondary lymphoid organs such as the spleen. We will describe these two experimental models in more details in relevant Results sections below.

EXPERIMENTAL PROCEDURES

Mice—$p27^{T187A/T187A}$ mice were described previously (11). Wild type and $p27^{T187A/T187A}$ mice were on mixed FVB and C57BL6J background. Mice were maintained under pathogen-free conditions in the Albert Einstein College of Medicine animal facility, and genotyped as previously described (19). Mouse experiments protocols were reviewed and approved by Einstein Animal Care and Use Committee, conforming to accepted standards of humane animal care.

Sheep Red Blood Cells (SRBC) Immunization—10–12-week-old mice (young mice) and 15–20-month-old mice (older mice) were immunized with 2×10^8 SRBC (Remel, R54012) by intraperitoneal injection. Spleens and sera were collected on day 10 for analyses.

Isolation of Splenocytes—Pieces of spleens were minced and passed through 70 μm cell strainers using the rod of 1 ml syringe into a 6-well plate in PBS. The strainers were washed with 1–2 ml of PBS. Cells were collected into a 15 ml tube and centrifuged at 1300 rpm for 5 min. Cell pellets were resuspended with 3–5 ml of red blood cell lysis buffer (155 mM NH_4Cl, 12 mM $NaHCO_3$, and 0.1 mM EDTA) and incubated at room temperature for 5 min. Then 10 ml of PBS+10%FCS was added to stop lysis, and splenocytes were collected by centrifugation.

Flow Cytometry (FACS)—5×10^6 to 1×10^7 isolated splenocytes were stained with fluorochrome-labeled antibodies at 4 °C for 30 min followed by washing with PBS/1% BSA, using our previously published protocol (20). Five-color flow cytometry was performed using an LSR II instrument (BD Biosciences) and the data were analyzed using FlowJo software (Tree Star, Inc.). At least 10,000 gated events were analyzed per mouse. For germinal center B cell analysis, isolated splenocytes were stained with DAPI (Sigma, D9564), Alexa 700-anti-B220 (BioLegend, 103231), FITC-PNA (Vector Labs, FL-1071), and PE anti-CD95 (Fas) (eBioscience, 12–0951-81). For T cell and B cell ratios analyses and CD4 and CD8 T cell ratios analyses, isolated splenocytes were stained with PE-anti-CD3 (BioLegend, 100205), Alexa 700-anti-B220 (BioLegend, 103231), Alexa 488-anti-CD4 (BioLegend,100425), and Alexa 647-anti-CD8a (BioLegend, 100727). Splenocytes were also stained with single fluorochrome-labeled antibody and used for compensation analyses.

Serum Anti-SRBC IgG ELISA—96 well EIA/RIA plates (Corning Inc., 9018) were coated for 1 h at 37 °C with either 100 μg/ml of SRBC ghosts prepared from sheep red blood cells (Thermo Fisher Scientific, R54012) for detecting antibodies in sera or mouse anti-goat IgG (H+L) (Southern Biotech, 1031-01) was used as plate coat to generate standard curve. The coated wells were blocked with 100 μl/well of 2% BSA/TBS for 1 h at 37 °C. Sera from unimmunized or immunized mice were serially diluted into 96 well plates in triplicate, and incubated overnight at 4 °C. After washed four times with TBST and bang dried, 96-well plates were added with 50 μl/well of alkaline phosphatase-labeled secondary antibody, goat anti-mouse IgG-AP (Southern Biotech, 1030-04), for 1 h at 37 °C. Alkaline

phosphatase substrate (0.5 mg/ml PNPP (Sigma, S0942)) in diethenolamine buffer (Fisher Scientific, 50-255-870) was then added to each well, and the plates were incubated 1 h at 25 °C before absorbance at 405 nm was measured in an ELISA reader. Serial dilutions and standard curves were performed to calculate antibody concentrations using Excel. Log transformation of concentrations was performed to obtain linear correlation between OD value and $\log_{10}$[concentration]. Purified isotype controls for mouse IgG1, IgG2a, IgG2b, and IgG3 (Southern Biotech) served as standards. Column scatter plots were generated by GraphPad Prism 6 software.

GC Number and Size Measurement—Overlapping images of entire spleen cross sections were reconstructed using "photomerge" (Adobe Photoshop CS3). Areas of entire spleen cross sections were used to determine GC numbers per mm^2. Sizes of individual GCs and spleen cross sections were measured in Image J. Column scatter plot of GC size was generated by GraphPad Prism 6 software; bar graph of GC numbers by Excel.

Tissue Sections and Staining—Pituitary glands and spleens were fixed in 10% formalin (Fisher Scientific, SF 100-4), embedded in paraffin wax and sectioned. For immunohistochemistry (IHC) and immunofluorescence (IF) staining, slides were deparaffinized, hydrated, and incubated in a steamer for 20 min in sodium citrate buffer (Vector Labs, H3301) for antigen retrieval. The following antibodies were used: anti-p27 (Abcam, ab92741), anti-CD3 (Santa Cruz Biotechnology, SC-20047), anti-B220 (BD Pharmingen, 550286), Ki67 (Vector Labs, SP6), anti-phosphorylated-Histon H3 (Cell Signaling Technology, #9701L), FITC-PNA (Vector labs, FL-1071), and Biotinylated PNA (Vector Labs, B-1075). SuperPicture™ kit (Invitrogen, 879263) and DAB kit with or without addition of nickel (Vector Labs, SK-4100) was used to detect p27 and Ki67 signals. Biotinylated anti-mouse (Vector Labs, BA-2000) and anti-rat (Vector Labs, BA-4000) antibodies were used as secondary antibodies for IHC CD3 and B220 staining, respectively, which were followed by Vectastain ABC kit (Vector Labs, PK-4004), and Red substrate kit (Vector Labs, SK-5100) for further red signal development. Biotinylated PNA was detected by Red substrate kit (Vector Labs, SK-5100). IF detection of pHH3, B220, CD3, and p27 was done by TSA™PLUS Fluorescence Kit (NEL741001KT, PerkinElmer), and PNA was by FITC-conjugated horse-anti-mouse-IgG (Vector Labs, FI-2000). DNA was stained with DAPI (Sigma, D9564). TUNEL staining was performed with an Apoptosis Detection Kit (Millipore, S7100).

Statistical Analysis—Four groups (young mice and older mice, each separated into WT and p27T187A KI genotypes) of data from ELISA and GC B cell FACS (Figs. 2 and 3, respectively) were analyzed by two-way (ages and genotypes) ANOVA (GraphPad Prism 6 Software), followed by unpaired two-tailed Student's *t* test (GraphPad Prism 6 Software) for pairs of WT and p27T187A in each age groups and pairs of young and older mice for each genotypes. Two groups (older WT and older p27T187A KI) of data from GC numbers and sizes measurements and pHH3 positive cell frequency in GC, and ratios of T/B cells and CD4+/CD8+ T cells (Figs. 4 and 7, respectively) were analyzed by unpaired two-tailed Student's *t* test (GraphPad Prism 6 Software). $p < 0.05$ is considered statistically significant.

RESULTS

p27T187A KI Prevents $Rb1^{+/-}$ Mice from Developing Pituitary Tumors—Study of retinoblastomas led to the identification of the prototype tumor suppressor pRb. Typical retinoblastoma patients inherit one null allele of *RB1* from one parent and therefore are $RB1^{+/-}$, they develop retinoblastoma with full penetrance and the tumors invariably are $RB1^{-/-}$. These characteristics led to the two-hit hypothesis for tumorigenesis by inactivation of a tumor suppressor. pRb is highly conserved in mouse (*Rb1* in mouse) and $Rb1^{+/-}$ mice develop melanotroph tumors in pituitary IL with full penetrance and the tumors are invariably $Rb1^{-/-}$ (21). We used this physiological setting to test $p27^{T187A/T187A}$ mice in oncogenic proliferation.

As expected, all ($n = 5$) $Rb1^{+/-}$ mice contained pituitary IL tumors when examined at 6 months of age. In comparison, all $Rb1^{+/-}$;$p27^{T187A/T187A}$ mice ($n = 5$) contained microscopically normal pituitary glands at 9 months of age, indistinguishable from pituitaries in wild type and $p27^{T187A/T187A}$ mice (Fig. 1*A*).

When measured by p27 IHC, expression of p27 in IL is low in WT, *Skp2* KO, and *POMC-Cre;*$Rb1^{lox/lox}$ (which deletes *Rb1* in all melanotrophs in IL) mice. Only when *Skp2* and *Trp53* were co-deleted in IL did p27 protein levels rise to produce robust staining, since co-deletion of *Skp2* and *Trp53* reduced the cellular pool of p27 ubiquitin ligases including Skp2, Pirh2, and KPC1 (22). Fig. 1*B* shows that p27 IHC produced similarly low staining in WT and $p27^{T187A/T187A}$ IL; tumors in $Rb1^{+/-}$ IL showed higher nuclear density but individual nuclei showed similar staining intensity with those in areas of normal IL; and $Rb1^{+/-}$;$p27^{T187A/T187A}$ IL was not different from $p27^{T187A/T187A}$ IL. In the same experiment, the IL of *POMC-Cre;*$Rb1^{lox/lox}$;*Trp53*$^{lox/lox}$;$Skp2^{-/-}$ mice showed robust staining (Fig. 1*C*).

These results document that blocking phosphorylation of p27T187 and, by extension, preventing p27 ubiquitination by $SCF^{Skp2/Cks1}$ does not result in overt accumulation of p27 protein in pituitary IL in adult mice, but can prevent pituitary IL tumorigenesis by two hit loss of *Rb1*.

$p27^{T187A/T187A}$ Mice Develop Normal T Cell-dependent Antibody Response that Does Not Decline with Age—Immunization with T cell dependent antigens, such as SRBC, induces robust antibody response that is largely dependent upon the formation of GCs in secondary lymphoid tissues (humoral immunity). The GC reaction is characterized by dramatic clonal expansion of GC B cells and selection of affinity matured subclones into either plasma cell or memory B cell pools. These dynamic changes in the GC B cell population are orchestrated by CD4+ follicular helper T cells (Tfh) in GC (23, 24). The GC response provides a physiological setting of robust but non-oncogenic proliferation to study cell cycle regulation (20, 25). In addition, both the magnitude and functional output of GC reaction decline in old age, a phenomenon contributing to increased incidence of vaccine-preventable diseases in the elderly population (26). We hypothesized that $p27^{T187A/T187A}$ mice, especially in older age, wound show impaired T cell-dependent antibody response, and set out to test our hypothesis.

In unimmunized mice, serum anti-SRBC IgG levels were at base levels in all test groups (Fig. 2*A*). We immunized 12-week-old WT and $p27^{T187A/T187A}$ mice (10–12-week-old mice will be

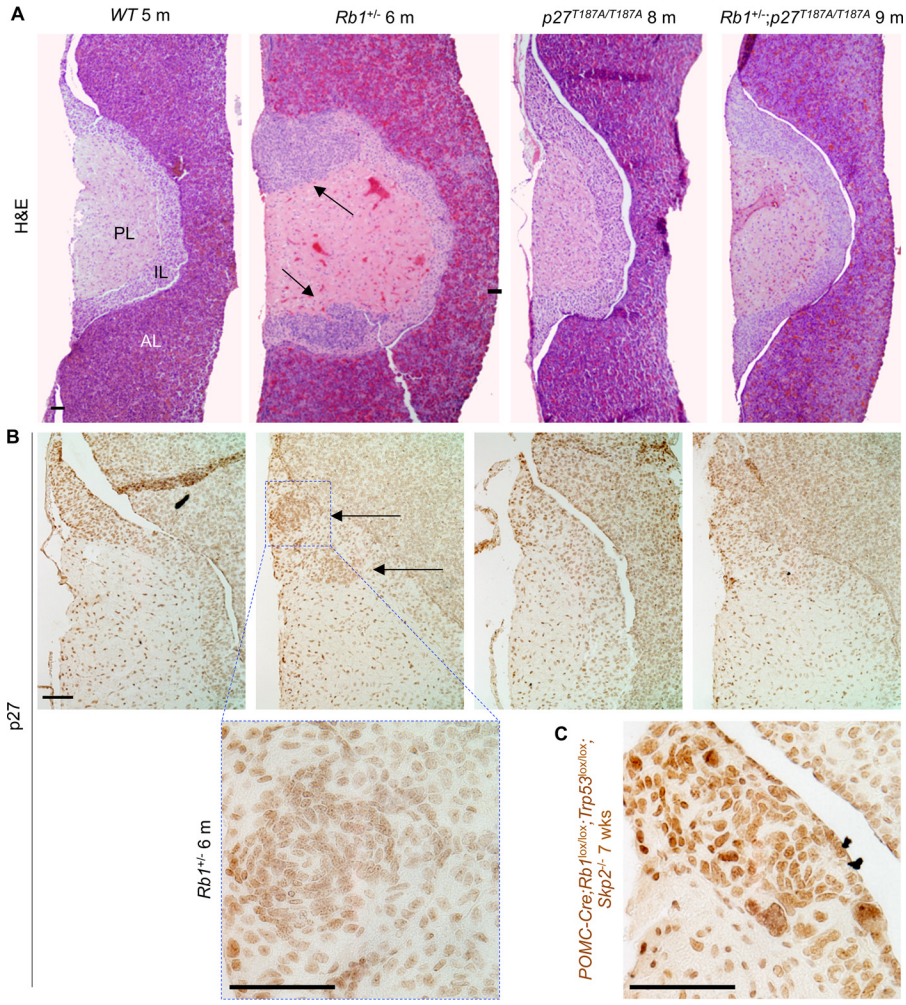


FIGURE 1. **Rb1$^{+/-}$;p27$^{T187A/T187A}$ mice do not develop pituitary tumors.** *A*, representative coronal sections of mouse pituitaries were stained with H&E. The three pituitary lobes are marked. *PL*, posterior lobe; *IL*, intermediate lobe; *AL*, anterior lobe. *Rb1*$^{+/-}$ mice at 6 months of age ($n = 5$) contained one to two IL tumors as marked by *black arrows*, while *Rb1*$^{+/-}$,*p27*$^{T187A/T187A}$ mice at 9 months of age ($n = 5$) contained pituitaries identical to those in WT and *p27*$^{T187A/T187A}$ mice. *B*, representative p27 IHC sections showing low level staining in WT, *Rb1*$^{+/-}$, *p27*$^{T187A/T187A}$, and *Rb1*$^{+/-}$;*p27*$^{T187A/T187A}$ IL, under the H&E images of the same genotypes. *C*, in the same experiment, *POMC-Cre;Rb1*$^{lox/lox}$;*Trp53*$^{lox/lox}$;*Skp2*$^{-/-}$ IL showed robust p27 staining. Scale bars, 100 μm.

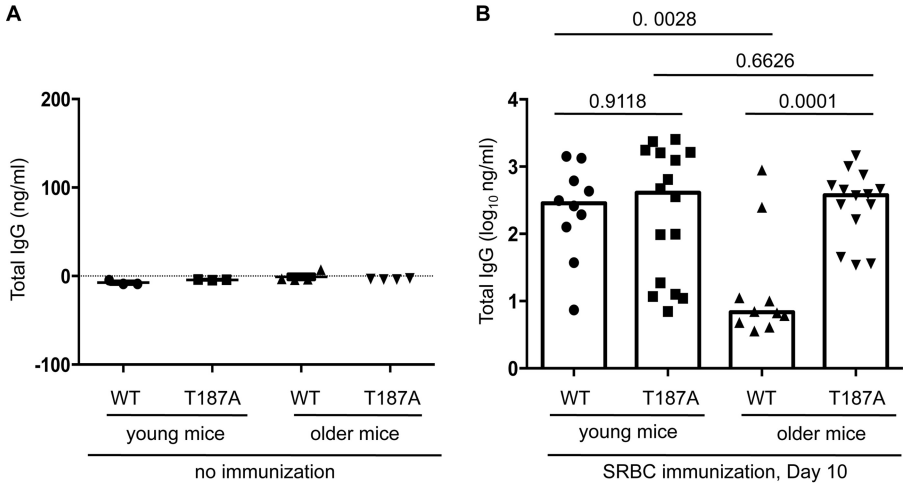


FIGURE 2. **Serum anti-SRBC IgG titers following SRBC immunization decline with age in WT but not *p27*$^{T187A/T187A}$ mice.** *A*, unimmunized WT or *p27*$^{T187A/T187A}$, young or older mice show similar base level serum anti-SRBC IgG titer. *B*, serum anti-SRBC IgG was measured 10 days after SRBC immunization. *WT* represents *p27*$^{+/+}$, *T187A* for *p27*$^{T187A/T187A}$. *young mice* were between 10 and 12 weeks old; *older mice* between 15 and 20 months old. Each data point represents one individual, and column heights show medians. Statistical analysis by two-way ANOVA with these four groups of data showed $p = 0.0129$ for differences between young and older, and $p = 0.0036$ for differences between WT and T187A. Unpaired two-tailed Student's *t* tests were used to determine *p* values between various pairs of samples as marked in the plot.

called "young mice") with SRBC and, on day 10, measured serum titers of anti-SRBC IgG. We found no significant differences between serum titers of anti-SRBC IgG in young WT and young $p27^{T187A/T187A}$ mice (Fig. 2*B*, *young mice*). Thus, p27T187A KI does not change the outcomes of SRBC immunization in young mice.

We went on to determine whether decline in humoral immunity would be greater in aging $p27^{T187A/T187A}$ mice than in aging WT mice. We found that anti-SRBC IgG titers reduced significantly in 15–20-month-old WT mice (15–20-month-old mice will be called "older mice") compared with young WT mice, but anti-SRBC titers in older $p27^{T187A/T187A}$ mice were not different from those in young $p27^{T187A/T187A}$ mice (Fig. 1*B*). As such, older $p27^{T187A/T187A}$ mice produced significantly higher titers of anti-SRBC IgG than older WT mice. Both two-way (ages and genotypes) ANOVA and Student's *t* test were performed for these analyses (see Fig. 2 legend). These findings proved opposite to our hypothesis, but phenotypically reveal that phosphorylation of p27T187 and, by extension, ubiquitination of p27 by $SCF^{Skp2/Cks1}$ promotes decline in humoral immunity in older mice.

Spleen GC Reactions Decline in Older WT, but Not Older $p27^{T187A/T187A}$, Mice—Antigen-specific antibody titers reflect the magnitude and quality of the GC response, in which naïve resting B cells convert to highly proliferating GC B cells. To determine the bases for the higher antibody titer in immunized older $p27^{T187A/T187A}$ mice, we determined how much B cell in total B cell population had converted to GC B cells at day 10 following SRBC immunization of four test groups of mice (as plotted in Fig. 3*D*). B cells are identified by B cell marker B220. GC B cells have additionally become positive for peanut agglutinin (PNA) affinity-staining and anti-Fas immune-staining. Spleen cell suspensions prepared on day 10 following SRBC injection were first gated on side and forward scatter to exclude aggregates and then for high B220 staining (for total B cells) and low DAPI staining (for total live B cells) (Fig. 3*A*). Within the total live B cell populations, GC B cell populations were identified as positive for both PNA and Fas. As shown in Fig. 3, *B* and *C*, and quantified in Fig. 3*D*, GC B cell populations ranged from 0.1% to 0.8% of the total B cell populations in non-immunized young and older, WT, and $p27^{T187A/T187A}$ mice. Following SRBC immunization GC B cell populations within total B cell populations increased in young WT and young $p27^{T187A/T187A}$ mice to 4.6% and 6.1%, respectively. With aging, GC B cell populations within total B cell populations declined in older WT mice (from 4.6% to 2.1%), but not in older $p27^{T187A/T187A}$ mice (from 6.1% to 6.3%). When older mice are compared, total B cell populations of older $p27^{T187A/T187A}$ mice contained significantly more GC B cells than older WT mice's total B cell populations. Both two-way (ages and genotypes) ANOVA and Student's *t* test were performed for the four groups of data (see Fig. 3*D* and its legend).

To directly visualize the GC structure, we stained spleen sections from immunized animals with PNA, and defined the PNA positive regions within the primary B cell follicles as GCs. p27T187A KI did not alter the spleen red pulp or white pulp morphology, nor did it cause spontaneous GCs in unimmunized animals of all age groups (not shown). After SRBC immunization, however, older $p27^{T187A/T187A}$ spleens developed many large GCs and often contained several GCs in each primary follicle compared with small to medium sized GCs each located within one follicle in the WT spleens (Fig. 4*A*). We quantified the GC density and size for individual spleen sections among the four experimental groups, and the results show that older $p27^{T187A/T187A}$ mice contained more numerous and larger GCs than older WT mice (Fig. 4, *B* and *C*). These results support the findings by FACS that GC B cell population sizes were larger in older $p27^{T187A/T187A}$ mice than in older WT mice (Fig. 3). A more robust GC reaction in older $p27^{T187A/T187A}$ mice could explain their improved anti-SRBC IgG titers relative to their WT counterparts.

We next examined the proliferation status of GC B cells using immune-staining for Ki67. Ki67 staining appeared similarly intense between older WT and young WT mice and between older WT and older $p27^{T187A/T187A}$ mice (Fig. 4*D* and data not shown). Although GCs in older WT mice are smaller than GCs in older $p27^{T187A/T187A}$ mice, nearly all PNA positive cells stained positive for Ki67 in both types of older mice (Fig. 4*D*). When GC B cells were stained with the mitotic marker phosphorylated histone H3 (pHH3), we found more pHH3 positive cells in GCs in older $p27^{T187A/T187A}$ mice than in older WT mice (Fig. 4, *E* and *F*), suggesting that more cell division could explain the larger GC B cell population sizes in older $p27^{T187A/T187A}$ mice. However, the increases did not reach statistical significance (Fig. 4*F*). TUNEL staining was similarly intense in older WT mice, young WT mice, and older $p27^{T187A/T187A}$ mice (data not shown). Thus, both the decline in older WT mice and the maintenance in older $p27^{T187A/T187A}$ mice of GC B cell population sizes were not correlated with dramatic decreases (in the decline in older WT mice) or increases (in the maintenance in older $p27^{T187A/T187A}$ mice) in Ki67 or pHH3 labeling of GC B cells.

In aggregate, by showing more robust production of GC B cells in older $p27^{T187A/T187A}$ mice than in older WT mice, results in this section explained improved serum titers of anti-SRBC IgG in older $p27^{T187A/T187A}$ mice. These findings however do not explain how p27T187A KI induced more robust GC B cell production.

p27 Expression Decreases When Naïve B Cells Are Recruited into GC in Both Older WT and Older $p27^{T187A/T187A}$ Mice—We next compared p27 expression in the spleens of older WT and older $p27^{T187A/T187A}$ mice at day 10 following SRBC injection, when the GC reactions were more robust in older $p27^{T187A/T187A}$ mice than in older WT mice.

In post-immunization spleens from both older WT and older $p27^{T187A/T187A}$ mice, the B220+ B cell area and CD3+ T cell zone occupy discrete areas in the white pulp (Fig. 5, *A*, *a*,*e* and *B*, *a*,*e*). The PNA+B220weak GCs were embraced by the PNA-B220+ primary B cell follicles from one side (Fig. 5*A*, *a*–*h*) with its other side likely facing the more discrete T cell zone (27, 28) (Fig. 5*B*, *a*–*h*).

Interestingly, the pattern of p27 staining is much broader than either the B220- or CD3-marked areas alone, suggesting that p27 is expressed in the majority of B and T cells outside of the GCs (also see below), and similar in older WT and older $p27^{T187A/T187A}$ spleen sections, suggesting that T187A KI did

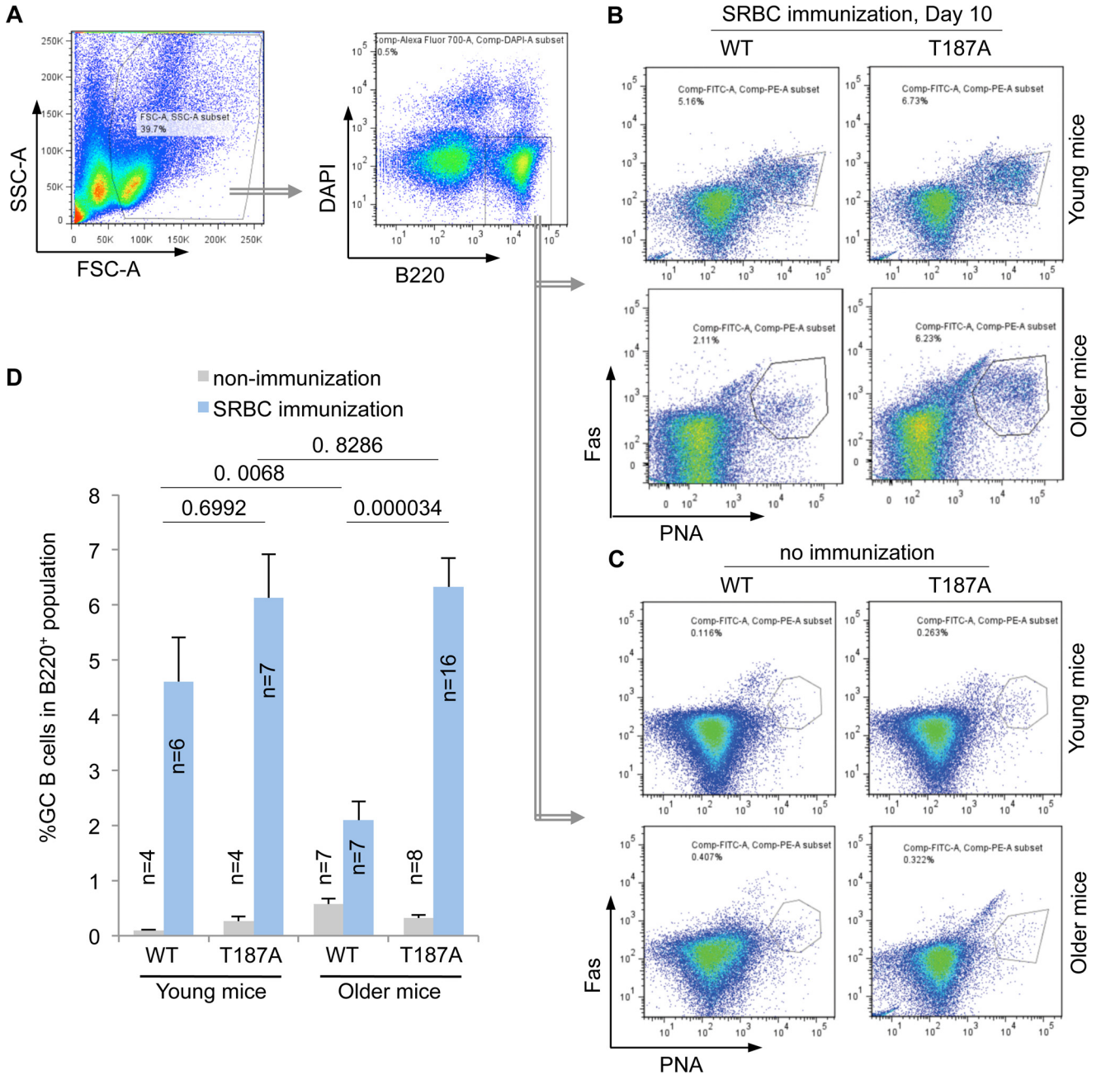


FIGURE 3. **Production of spleen GC B cells following SRBC immunization decline with age in WT but not *p27*$^{T187A/T187A}$ mice.** *A*, FACS of total spleen cell suspension, showing the first FSC-SSC gate, followed by the B220 and DAPI gate. DAPI^{low},B220^{high} cells then underwent FACS on PNA and Fas to determine population sizes of GC B cells (PNA^{high} and Fas^{high}) in total B cell population at day 10 following SRBC immunization (*B*) or from unimmunized mice (*C*). Quantifications of *B* and *C* are shown in *D*. Error bars represent S.E. Two-way ANOVA with these four groups of data from immunized mice showed $p = 0.0850$ for differences between young and older, and $p = 0.0001$ for differences between WT and T187A. Unpaired two-tailed Student's *t* tests were used to determine *p* values between various sample pairs as shown in *D*.

not induce notable p27 accumulation in *p27*$^{T187A/T187A}$ spleens (Fig. 5*C*, *a* and *e*). Notable however are round punctuates where p27 staining was dramatically reduced (*black arrows* in Fig. 5*C*, *a* and *e*) inside the broad and otherwise evenly stained p27+ areas in both WT and *p27*$^{T187A/T187A}$ spleens. Double immunofluorescence (IF) staining with PNA and anti-p27 demonstrate that the p27 low areas are populated by PNA+ GC B cells (*white arrows* in Fig. 5, *c*,*d* and *g*,*h*). High magnification images confirmed that the vast majority of PNA+ GC B cells are p27 negative irrespective of the p27 genotype (Fig. 6*A*). Thus, in the B cell lineage, p27 protein levels were dramatically down regulated when the naïve resting B cells (which populate the more intensely stained B220+ area) are recruited into the highly proliferative GCs regardless of the *p27*$^{T187A/T187A}$ mutation status.

Since GC B cells were actively proliferating in both older WT and older *p27*$^{T187A/T187A}$ mice (Fig. 4, *D* and *E*), the dramatic down-regulation of p27 in GC correlated with highly active proliferation of GC B cells. To identify mechanisms for this down-regulation in GC B cells, we determined whether p27 mRNA levels were down regulated in GC B cells. For this purpose, we searched NCBI databases for gene expression profile datasets that compared naïve resting B cells with GC B cells. We

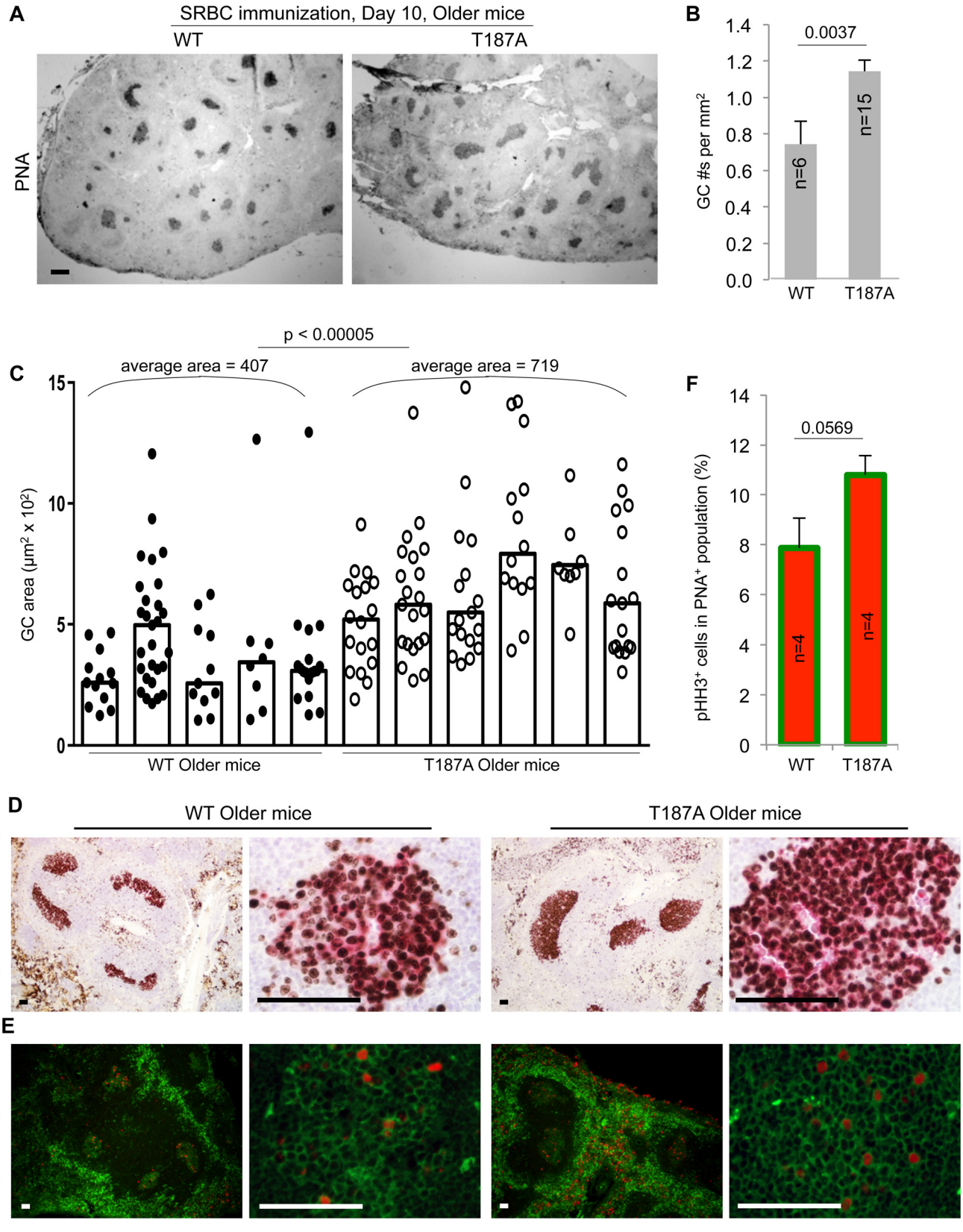
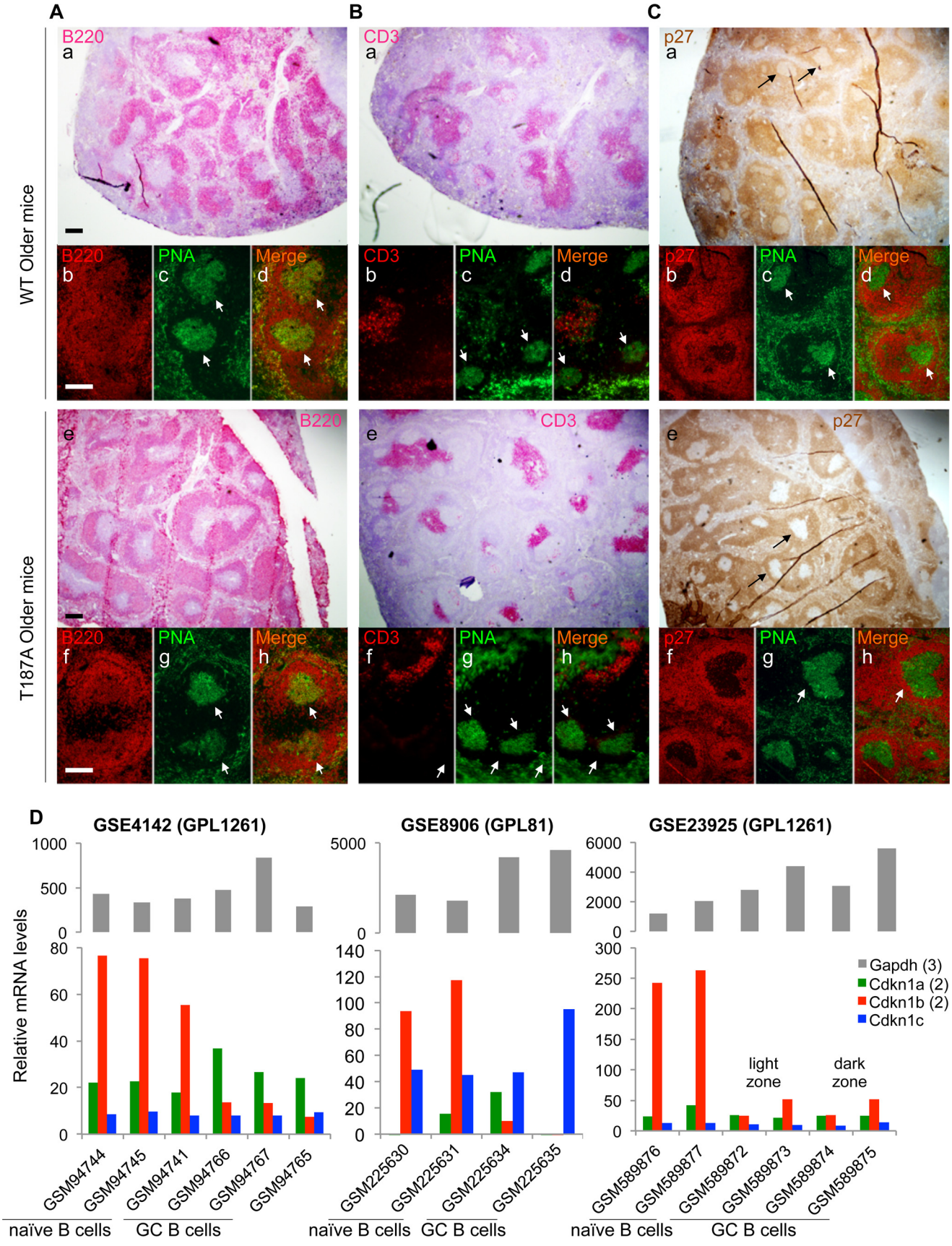


FIGURE 4. **Spleen GC numbers and sizes are higher and larger in older *p27*$^{T187A/T187A}$ mice than in older WT mice at 10 days following SRBC immunization.** *A*, representative images of spleen sections stained with PNA to visualize GC. Scale bars, 200 μm. *B*, quantifications of GC numbers on spleen sections, error bars are S.E. *C*, quantifications of GC sizes. Each column represents one individual, and each point is an individual GC. Column heights represent medians. Co-staining of Ki67 (*black*) and PNA (*red*) (*D*), and pHH3 (*red*) and PNA (*green*) (*E*), of GC were performed on spleen sections as marked. Scale bars, 50 μm. *F*, quantification of data in *E*. *p* values are from two-tailed Student's *t* tests.



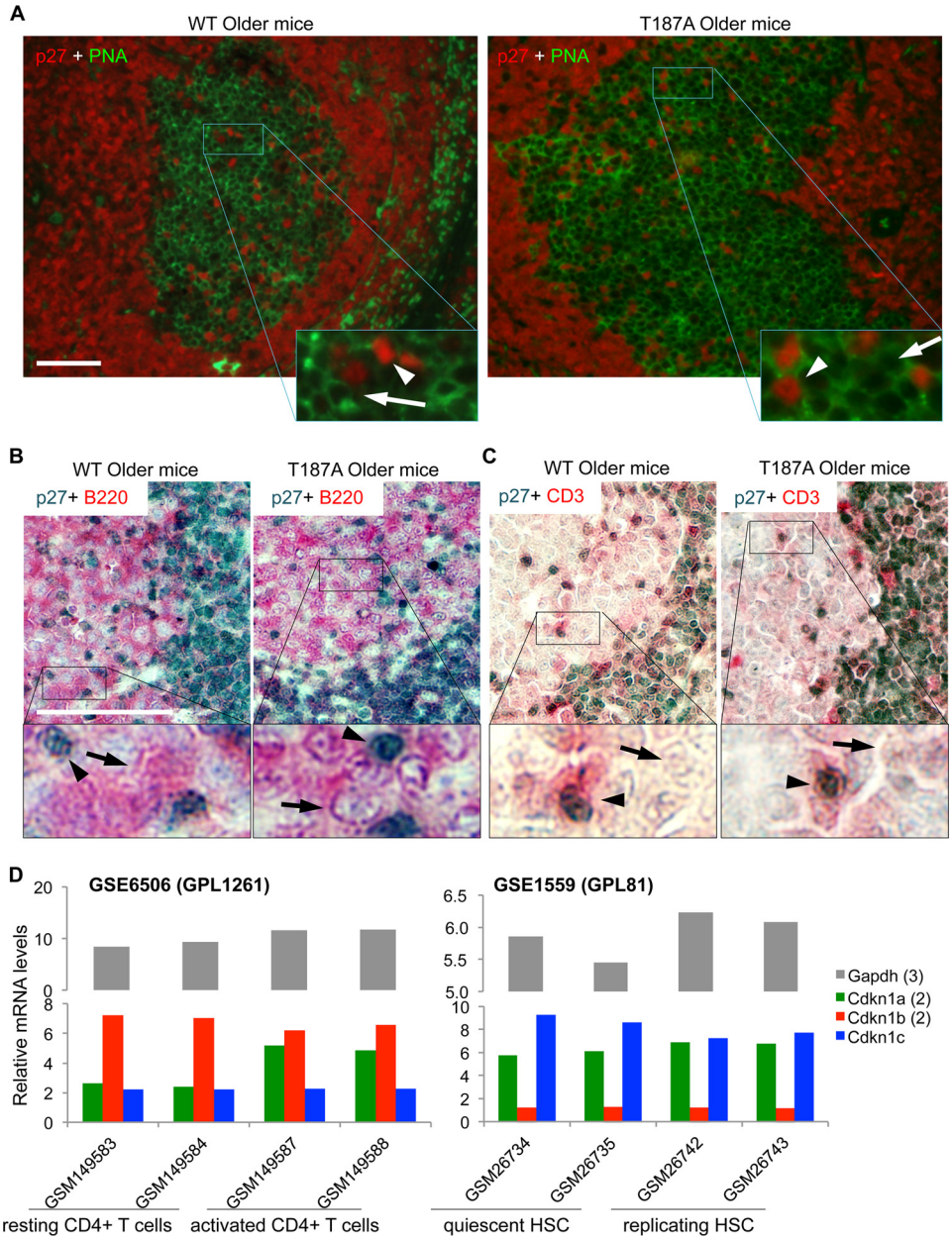


FIGURE 6. **p27 expression is dramatically reduced in GC B cells but maintained in GC T cells in older WT and older *p27*$^{T187A/T187A}$ mice at 10 days following SRBC immunization.** *A*, high magnification and red-green channel merge images of co-IF for p27 and PNA, as indicated, of representative GC, as marked. In enlarged areas, *white arrows* point to cells with membrane staining by PNA but negative for p27, while *white arrowheads* point to cells with positive staining for p27 without membrane PNA staining. *B*, two-color co-IHC for p27 (*dark blue*) and B cell marker B220 (*pink*), as marked, showing approximately one-quarter of a GC. *Black arrows* point to cells with positive membrane staining for B220 but negative for p27, while *black arrowheads* point to cells with positive p27 staining but negative for B220 membrane staining. *C*, same experiments as in *B*, except B220 antibody was replaced with CD3 antibody. *D*, in NCBI GEO database, gene expression profiles for naïve and activated CD4+ T cells (GSE6506) and quiescent and replicating HSC (GSE1559) were queried for expression of p21 (Cdkn1a, average of 2 probe sets), p27 (Cdkn1b, average of 2 probe sets), and p57 (Cdkn1c), as well as a housekeeping gene Gapdh (average of 3 probe sets). Sample records (GSMxxx) and experimental platforms (GPLxxx) are shown. Data in both datasets were normalized by GC-RMA, and two biological replicates were analyzed.

found three such datasets and queried them for expression of the three p27 family members including p21 (Cdkn1a), p27 (Cdkn1b), and p57 (Cdkn1c) together with a housekeeping gene *Gapdh*. As shown in Fig. 5*D*, p27 mRNA levels were reduced by 5.5 to 10.5-fold in GSE4142, 117 to 9.5-fold in GSE8906, or 4.7 to 10.6-fold in GSE23925 when naïve B cells

FIGURE 5. **p27 expression is reduced in GC in both older WT and older *p27*$^{T187A/T187A}$ mice.** Spleen samples were collected 10 days following SRBC immunization, and stained by IHC with anti-B220 (*A, a* and *e*), anti-CD3 (*B, a* and *e*), or anti-p27 (*C, a* and *e*). Co-IF was then performed with PNA and each of these three antibodies to identify areas of GC, pointed by *white arrows*, in stains by each of these three antibodies (*b,c,d* and *f,g,h panels*). Scale bars, 200 μm. *D*, three gene expression profile databases (NCBI GEO accession numbers, GSExxx, are as marked) were queried for expression of p27 family members p21 (Cdkn1a, average of 2 probe sets), p27 (Cdkn1b, average of 2 probe sets), and p57 (Cdkn1c), as well as a housekeeping gene Gapdh (average of 3 probe sets). Sample records (GSMxxx) obtained from purified naïve B cells and GC B cells are as marked. Accession numbers GPLxxx contain information on experimental platforms (Affymatrix microarrays). Data in GSE4142 were RMA (Robust Multi-array Average) normalized unlogged, and GSE23925 was normalized by RMA. GSE8906 contained raw data. Two to three biological replicates were analyzed.

were compared with GC B cells. In the same samples, p21 and p57, as well as Gapdh, mRNA levels showed no such reduction patterns.

These findings suggest the activation of a robust mechanism that inhibited p27 mRNA expression when naïve B cells are stimulated to enter GC to proliferate. The dramatic p27 mRNA down-regulation might have overridden any reduction in p27 protein degradation caused by p27T187A KI, resulting in dramatic p27 protein level reductions in GC B cells in both WT and $p27^{T187A/T187A}$ mice, as shown in Fig. 5*C*. With this line of reasoning, we conclude that the enhanced GC reaction in older T187A mice is not caused by p27T187A KI cell-autonomously in GC B cells.

GC T Cells Maintain p27 Protein Levels—Because GC B cells dramatically down-regulate both p27 mRNA and protein expression, any effects of the p27T187A KI on p27 protein degradation might be inconsequential in GC B cells. We reasoned that cells in GC that maintain p27 protein levels are more likely to be affected by p27T187A KI. Although B cells outnumber T cells within GC, the CD4+ helper T cells in GC play a critical role in all aspects of the GC response including its initiation, expansion, and termination (24). As such, we next focused our p27 protein expression study on GC T cells. High magnification images of p27 and PNA co-IF indeed revealed a number of p27-positive cells in GC (Fig. 6*A*), with the vast majority of the p27-positive cells stained negative for PNA (*white arrowheads*), indicating that they are not B cells.

To determine the lineage status of the p27 expressing cells in GC, we concurrently stained p27 with either B220 or CD3 in double IHC. Consistent with co-IF for p27 and PNA in Fig. 6*A*, p27 positive cells in GC are negative for B220 in co-IHC (Fig. 6*B*, *black arrowheads*), while most cells in GC show positive B220 membrane staining with negative p27 staining (*black arrows*). In contrast, p27 positive cells in GC are positive for CD3 in co-IHC while most cells in GC are negative for both p27 and CD3 (Fig. 6*C*, *black arrowheads* and *black arrows*, respectively). These findings demonstrate that, while GC B cells dramatically down regulate p27 protein expression, GC T cells maintain p27 protein expression. Based on the IHC technique, we could not demonstrate any difference in p27 protein levels between the WT and $p27^{T187A/T187A}$ GC T cells.

Rapid proliferation of GC B cells leading to the generation of GC in response to immunization by T cell dependent antigen is completely dependent on CD4+ T helper cells (29). Inhibition of T cell proliferation by transgenic expression of p27 in T cells (from the mouse *Lck* promoter) blocked spleen GC reactions (30). Decline in CD4+ T cell population with aging has been suggested as a cause for the decline in GC response to T cell-dependent antigen immunization in older WT mice (31, 32). And it is known that proliferation of splenic CD4+ T helper cells in response to stimulation by anti-TCR was significantly (80%) reduced by p27T187A KI (11).

Combining these previous knowledge with our current findings that GC B cells do not express p27 mRNA and protein but GC T cells maintain p27 protein expression led us to hypothesize that CD4+ T cells in young $p27^{T187A/T187A}$ mice might be more difficult to activate and therefore have undergone less accumulative proliferation to have conserved a larger proliferative reservoir to maintain robust GC responses in older $p27^{T187A/T187A}$ mice. This principle is well known in stem cell studies (33, 34). In adult mice, hematopoietic stem cells (HSCs) remain proliferative quiescence until stimulated to proliferate to replenish the lost cell population. In resemblance, naïve CD4+ T cells are resting until activated by antigen binding to TCR to expand and migrate to GC to play essential roles in establishing humoral immunity (35, 36). In fact, it was shown in 2007 that CD4+ T cell activation and HSC activation shared both up-regulated and down-regulated gene expression programs (37). To determine whether regulation of p27 mRNA expression is also similar between CD4+ T cell activation and HSC activation, we queried NCBI GEO databases as shown in Fig. 6*D*. In GSE6506 (37) mouse splenic CD4+ T cells were activated by Concanavalin A and, in GSE1559 (38), mouse HSCs were activated by administering 5-fluorouracil. We found that p27 mRNA levels were not reduced when naïve CD4+ T cells were activated, consistent with the presence of p27 positive T cells in GC (Fig. 6*C*). Interestingly, p27 mRNA levels were also not reduced when HSCs were activated to replicate. These findings add a further relatedness between the activation programs in naïve CD4+ T cells and adult HSCs. Lack of p27 mRNA down-regulation could provide an opportunity for p27 down-regulation by p27 protein degradation.

Spleen CD4+/CD8+ T Cell Ratios following SRBC Immunization Is Higher in Older $p27^{T187A/T187A}$ Mice than in Older WT Mice—Altered cell proliferation control may lead to changes in clonal expansion, differentiation, and cell death. As an indirect measurement of the impact of p27 T187A KI on lymphocyte population maintenance, we assessed the population size of total T and B cells (as T/B cell ratios), and CD4+ and CD8+ T cell subsets (as CD4+/CD8+ T cell ratios) in older mice. As shown in Fig. 7*A*, the T/B cell ratios were comparable between WT and $p27^{T187A/T187A}$ mice. Interestingly, the ratio of CD4+ helper T cells over CD8+ killer T was significantly higher in older $p27^{T187A/T187A}$ mice compared with older WT mice (Fig. 7*B*). This finding suggests that a more difficult-to-activate CD4+ T cell population in $p27^{T187A/T187A}$ mice (11) might have better maintained its population size in older age, and could explain how older $p27^{T187A/T187A}$ mice responded more robustly than older WT mice to SRBC immunization. To strengthen this finding, we further determined the CD4+/CD8+ T cell ratios inside CD3+ gate, and obtained similar results (Fig. 7*C*). We conclude that older $p27^{T187A/T187A}$ mice contained larger populations of CD4+ T cells during SRBC immunization, which, at least in part, enhanced GC B cell reaction.

DISCUSSION

Modeling Effects of Skp2/Cks1-p27T187p Interaction Inhibitors—Roles of p27 in proliferation related physiology have been well studied with loss-of-function approaches using p27 KO mice and p27 ck- mice. Since increasing p27 is an actively developing cancer therapy strategy, studying effects of p27 gain-of-function in proliferation related physiology would more directly facilitate cancer therapy development. In this regard, $p27^{T187A/T187A}$ mice are especially important since they model actions of an actively developing type of inhibitors that block p27T187p interaction with Skp2/Cks1 (see Introduction). Fur-

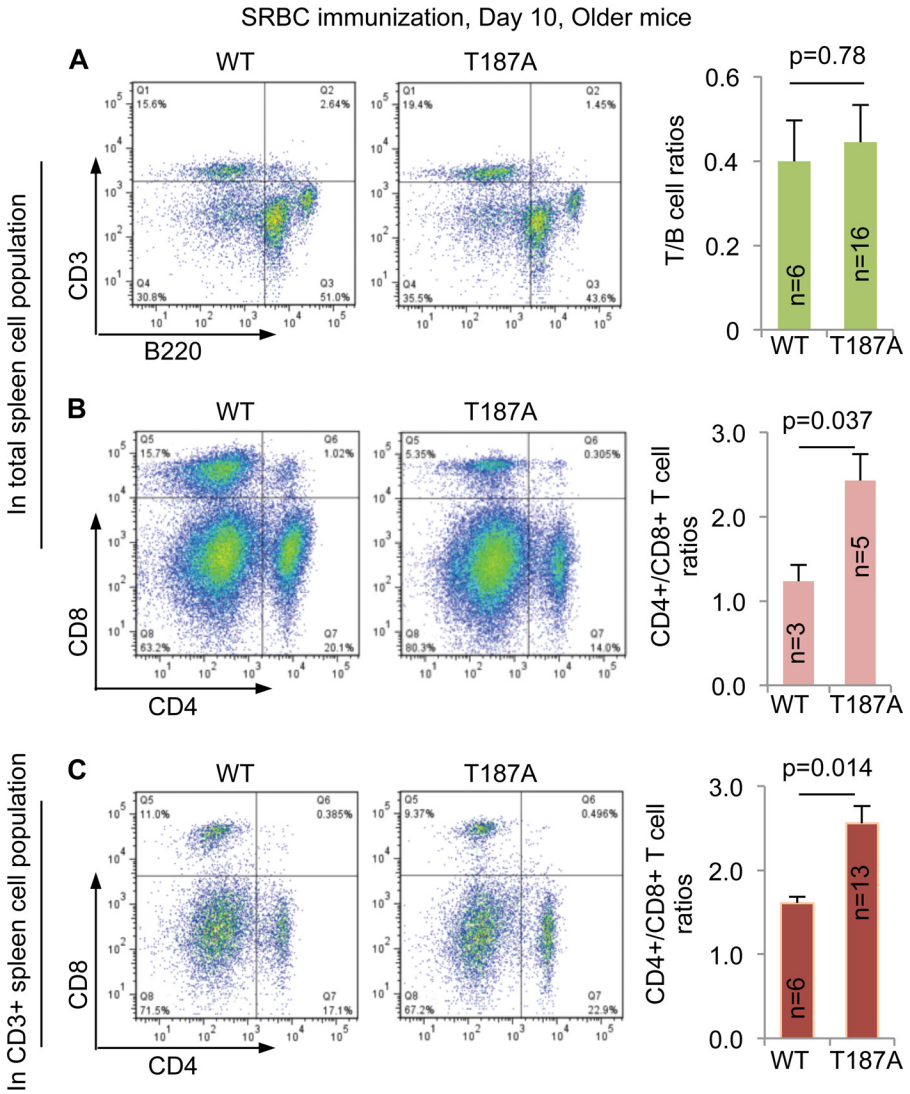


FIGURE 7. **CD4+/CD8+ T cell ratios are higher in older *p27$^{T187A/T187A}$* mice than in older WT mice at 10 days following SRBC immunization.** *A*, representative FACS profiles of spleen cell suspensions following CD3 and B220 double staining and quantifications. *B*, FACS profiles following CD4 and CD8 double staining of total spleen cell suspension and quantifications. *C*, FACS profiles following CD4 and CD8 double staining of CD3+ cells in spleen cell suspension and quantifications. *p* values are from two-tailed Student's *t* tests, and error bars represent S.E.

ther, *p27$^{T187A/T187A}$* mice do not accumulate p27 protein in quiescent tissues and their lack of overt defects already demonstrated that inhibitors of p27T187p interaction with Skp2/Cks1 should be harmless to normal physiology. Remarkably, since its generation and first characterization in 2001 (11), studies of *p27$^{T187A/T187A}$* mice have revealed few physiological phenotypes including tumorigenesis (see Introduction). Our study now reveal that fully penetrant pituitary tumorigenesis in *Rb1$^{+/-}$* mice is fully blocked in *Rb1$^{+/-}$;p27$^{T187A/T187A}$* mice, identifying *Rb1$^{+/-}$* mice as the preclinical tumor model against which inhibitors of p27T187p interaction with Skp2/Cks1 can be definitively compared.

p27T187A KI models the most potent inhibitors of p27T187p-Skp2/Cks1 interaction and, therefore, can be an impossible match for inhibitor candidates. On the other hand, inhibitors of p27T187p-Skp2/Cks1 interaction may also block interaction of Skp2-Cks1 to p27 family members p21 and any unknown substrates of SCF$^{Skp2/Cks1}$, potentially inhibiting pituitary tumorigenesis in *Rb1$^{+/-}$* mice through mechanisms other than stabilizing p27. More sophisticated preclinical mouse tumor models may be desired to improve on these aspects of inhibitor testing using *Rb1$^{+/-}$* mice.

Mechanisms of Tumor Blocking in Rb1$^{+/-}$;p27$^{T187A/T187A}$ Mice—In our previous studies, we induced mouse pituitary IL tumors by artificial deletion of *Rb1* in all melanotrophs and POMC-Cre;*Rb1*$^{lox/lox}$ mice developed pituitary tumors across the entire IL. POMC-Cre;*Rb1*$^{lox/lox}$;p27$^{T187A/T187A}$ mice produced greatly thinned pituitary IL with apoptosis (19). In this artificial setting, p27T187A KI resulted in synthetic lethality with *Rb1* deletion by further activating E2F1 (39). Our current finding documents a tumor suppressive role of p27T187A KI in a physiological "two hit" setting. Whether this block was achieved by promoting apoptosis or inhibiting proliferation is difficult to determine in the "two hit" setting. It is however likely that the observed blocking to pituitary tumorigenesis by two hit *Rb1* loss does not require significant accumulation of p27, since p27 IHC showed similarly low levels of p27 in WT, *p27$^{T187A/T187A}$*, and *Rb1$^{+/-}$;p27$^{T187A/T187A}$* IL (Fig. 1*B*).

It is notable that, p27T187A KI showed little antitumor effects in lung tumorigenesis by oncogenic activation of endogenous *Kras* (14), another tumor model in a physiological setting. Since p27 mRNA levels were significantly reduced in this lung tumor model (14), the lack of an antitumor phenotype demonstrates that the inhibitory effects of p27T187A KI on p27 protein degradation might only be relevant when p27 protein levels were reduced primarily by enhanced protein degradation. Differences in oncogenic mechanisms by two hit loss of *Rb1* in melanotrophs and by activation of endogenous *Kras* in lung may also determine whether p27T187A KI is effective. Now that the "positive control" is available (that $Rb1^{+/-}$; $p27^{T187A/T187A}$ mice do not develop pituitary tumors), testing antitumor effects of p27T187A KI in other physiological mouse tumor models will be more informative.

How Does p27T187A KI Maintain Robust Antibody Response to SRBC Immunization?—While blocking pituitary tumorigenesis by "two hit" loss of *Rb1* identifies a sought-after role for an anticipated effect, enhancing GC B cell expansion leading to improved antigen specific humoral immunity in older mice is opposite to our anticipation. This unexpected finding has the potential to address another important medical issue: improve vaccination efficacy in the elderly. Vaccination efficacy depends on long lived plasma cells and memory B cells, both of them produced by the GC reaction following immunization. Both B and T cell repertoires experience age-associated impairment in mice and humans. In co-transfer experiments testing different combination of old and young B and T cells, it was observed that the age-related GC impairment mainly engrafts with old T cells instead of old B cells (32, 40). More recent studies have revealed multiple, age-associated molecular changes in the follicular T helper cell lineage (Tfh cells, which are CD4+ T cells in GC) (26). Our findings have now implicated the regulation of p27 protein stability in these aspects.

We suggest that ubiquitination of p27T187p by $SCF^{Skp2/Cks1}$ in CD4+ T cells may have set a threshold for their activation at a level that could lead these cells to proliferate in young age to the extent that compromises their proliferation in older age. Indeed, there were more GCs in a given area of the spleen section in older $p27^{T187A/T187A}$ mice than older WT mice (Fig. 4*B*), suggesting increased availability of cognitive T cells to SRBC-reactive B cells to initiate the GC response in the mutant mice. In other words, the efficiency of T cell priming, which is normally reduced with aging (26, 29), was maintained by the expression of the p27T187A protein. In humans, reduced antibody response to vaccination correlates with reduced frequency of naïve T cells (41). Thus, it is possible that a stabilized p27 protein and the consequent reduced cell cycle activity upon antigen stimulation (11) preserved more proliferation potential of the naïve CD4+ T cell pool, while this pool is diminished with aging in WT mice. This notion is supported by our observation that older $p27^{T187A/T187A}$ mice had a higher CD4+/CD8+ T cell ratio relative to older WT mice (Fig. 7).

The second aspect of the observed enhanced GC phenotype is that individual GCs formed in older $p27^{T187A/T187A}$ mice was 77% larger on average than those in older WT mice (Fig. 4*C*), indicating that more mitogenic signals were provided by Tfh in GC to the GC B cells, which is another aspect of T cell function known to deteriorate with aging (26, 29). While detailed molecular mechanisms linking a stabilized p27 protein to enhanced B cell help function of GC T cells await future studies, it is worth noting that a recent study has correlated reduced proliferation of Tfh cells to their enhanced B cell help function (42).

In summary, our study have provided evidence that targeting p27T187 phosphorylation-dependent ubiquitination by $SCF^{Skp2/Cks1}$ could be very effective against specific types of cancer (43) and have suggested strategies to better understand, and potentially leading to methods to prevent, the decline of humoral immunity in the elderly (26).

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HHLA2 and TMIGD2: new immunotherapeutic targets of the B7 and CD28 families

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We and others recently discovered HHLA2 as a new B7 family member and transmembrane and immunoglobulin domain containing 2 (TMIGD2) as one of its receptors. Based on a new study we propose that HHLA2 may represent a novel immunosuppressive mechanism within the tumor microenvironment and hence could be a target for cancer therapy. TMIGD2 may be another therapeutic target.

HHLA2 (B7H7/B7-H5/B7y) has recently been identified as a new member of the B7 family member.[1-3] HHLA2 was initially discovered as a gene in the Immunoglobulin (Ig) superfamily when screening the human genome for human endogenous retroviral (HERV) long terminal repeat (LTR) sequences which provide polyadenation signals.[4] Hence the name, HHLA2, is short for HERV-H LTR-associating 2. HHLA2 orthologs appear to be present in a wide range of species such as fish, frog, giant panda, monkey and human, but not in laboratory mouse and rat strains. The HHLA2 protein has amino acid similarity of 23 to 33% to the other human B7 family molecules and phylogenetically it is most similar to B7-H3 and B7x (B7-H4/B7S1). The predicted structure of HHLA2 is a type I transmembrane molecule with three extracellular Ig domains. This is unique as most other B7 family members contain only two Ig domains while human B7-H3 has four Ig domains (**Fig. 1A**).

HHLA2 functions as a T cell coinhibitory molecule as it suppresses proliferation and cytokine production of both human $CD4^+$ and $CD8^+$ T cells.[1,5] HHLA2 is constitutively expressed on the surface of human monocytes and is induced on B cells after stimulation.[1] Unlike PD-L1 and B7–1 though, HHLA2 is not inducible on T cells. The differences in expression on immune cells suggest that HHLA2 could be involved in immune regulation at a different functional level than other B7 family members. Using immunohistochemistry with an HHLA2 monoclonal antibody, we have recently found that HHLA2 is not expressed in most human tissues, except the placenta, kidney, intestine, gall bladder, and breast.[6] Expression of HHLA2 in the placenta and the intestines is interesting as it may help fetal-maternal immune tolerance or control intestinal inflammation, respectively. Importantly, we have shown many human cancers overexpress HHLA2 including cancers from the breast, lung, thyroid, melanoma, pancreas, ovary, liver, bladder, colon, prostate, kidney, and esophagus.[6] Moreover, in a small cohort of human triple-negative breast cancer (TNBC) patients, higher expression of HHLA2 on tumor cells was associated with increased lymph node metastases.[6] The wide expression of HHLA2 in human cancers and its association with more invasive disease in the TNBC cohort suggest that HHLA2 potentially plays an important role in tumor evolution and metastases through immune suppression.

There are at least two mechanisms upregulating HHLA2 expression. One mechanism is inflammatory stimulation.[1,2] HHLA2 expression can be increased on monocytes and macrophages and is induced on B cells by stimulation with LPS and IFN-γ.[1,2] The second mechanism is the gene copy-number variation.[6] We compared gene dosage in the basal subtype of TNBC using the cBioPortal for the Cancer Genomics database.[7] In this subtype, 32% had HHLA2 gene copy-number variations and the majority (95%) of these variants were either amplifications or gains of HHLA2 gene copy number,[6] suggesting this could be another mechanism of overexpression.

Receptors for HHLA2 can be found on a wide variety of immune cells, including T cells, B cells, monocytes, and dendritic cells.[1] We and others have independently identified one of receptors for HHLA2, TMIGD2,[6] also called CD28 homolog (CD28H),[2] through a bioinformatics analysis/immunological approach and a high-throughput screening, respectively. Like HHLA2, this molecule is expressed in humans and monkeys but not in mice or rats. This molecule was initially reported as an endothelial adhesion molecule which was renamed Immunoglobulin-containing and Proline-rich

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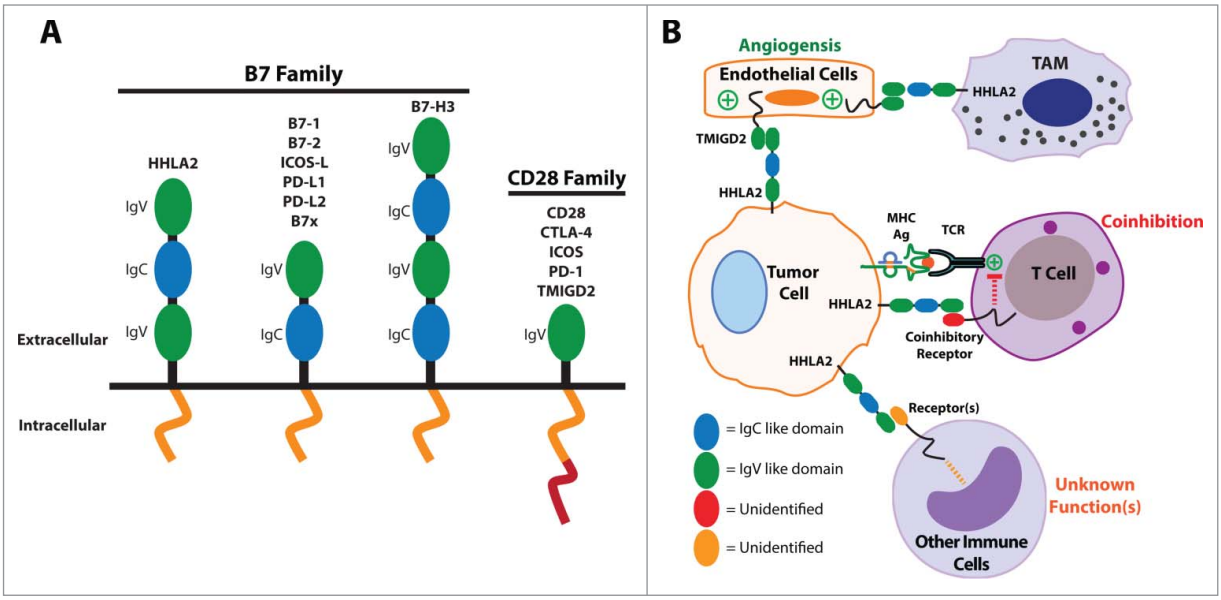

Figure 1. The B7 and CD28 families and the significance of HHLA2 and TMIGD2 within the tumor microenvironment. (**A**) A structural representation of the B7 and CD28 family members. (**B**) A proposed model for the roles of HHLA2 and TMIGD2 within the tumor microenvironment. Tumor-expressed HHLA2 can interact not only with an unidentified receptor on activated T cells that leads to coinhibition, but also with TMIGD2 on endothelium that stimulates tumor angiogenesis. Additionally, tumor-expressed HHLA2 can bind to other immune cells and likely affects their functions in ways that are not yet understood. Finally, tumor-associated macrophages (TAM) may express HHLA2 and interact with TMIGD2 on endothelium.

Receptor-1 (IGPR-1).[8] TMIGD2 protein can be detected in cells of epithelial and endothelial origins, and is able to enhance angiogenesis *in vitro* when overexpressed by endothelial cell lines.[8] Furthermore, TMIGD2 is reported as a stimulatory receptor expressed primarily on naive T cells.[2] Like other CD28 family members, TMIGD2 is an Ig superfamily member with an extracellular IgV-like domain, a transmembrane region, and a cytoplasmic tail.[6] The cytoplasmic tail contains tyrosine residues which can be phosphorylated[2] and a proline-rich domain which associates with multiple Src homology 3 (SH3)-containing signaling molecules.[8] Together, these studies suggest that TMIGD2 has multiple functions depending on the cell type and signaling pathways.

In summary, we have shown that the HHLA2 pathway could represents a novel immunosuppressive mechanism within the tumor microenvironment and is an attractive target for human cancer therapy. HHLA2's overexpression may be advantageous to cancer growth and survival through different mechanisms (**Fig. 1B**). Tumor-expressed HHLA2 could protect the tumor from immune surveillance via its interaction with unidentified receptors on activated T cells and other immune cells, and it may also promote angiogenesis within the microenvironment via its interaction with endothelial-expressed TMIGD2. The blockade of the B7–1/B7–2/CTLA-4 and PD-L1/PD-L2/PD-1 pathways within the B7 and CD28 families to enhance anti-tumor immunity has been exploited with therapeutic success.[9,10] Interestingly, therapies targeting HHLA2 could not only enhance anti-tumor immune responses, but may also inhibit tumor angiogenesis. Further studies are required to dissect TMIGD2's expression patterns and functions in order to develop new therapies targeting TMIGD2.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Review

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New immunotherapies targeting the PD-1 pathway

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Ligands from the B7 family bind to receptors of the CD28 family, which regulate early T cell activation in lymphoid organs and control inflammation and autoimmunity in peripheral tissues. Programmed death-1 (PD-1), a member of the CD28 family, is an inhibitory receptor on T cells and is responsible for their dysfunction in infectious diseases and cancers. The complex mechanisms controlling the expression and signaling of PD-1 and programmed death ligand 1 (PD-L1) are emerging. Recently completed and ongoing clinical trials that target these molecules have shown remarkable success by generating durable clinical responses in some cancer patients. In chronic viral infections, preclinical data reveal that targeting PD-1 and its ligands can improve T cell responses and virus clearance. There is also promise in stimulating this pathway for the treatment of autoimmune and inflammatory disorders.

Expression of PD-1 and its ligands PD-L1 and PD-L2

PD-1 (CD279) is an inhibitory receptor from the CD28 family that is expressed on various immune cells including T and B lymphocytes, dendritic cells (DCs), monocytes, and macrophages [1–4]. While PD-1 is not expressed on naïve T cells, it is upregulated following T cell receptor (TCR)-mediated activation and is readily observed on both activated and exhausted T cells [5,6]. These 'exhausted' T cells have a dysfunctional phenotype and are unable to respond appropriately to stimuli. Although PD-1 has a relatively wide expression pattern, its most important role is likely as a coinhibitory receptor on T cells. Current therapeutic approaches focus on blocking the interaction of this receptor with its ligands to enhance T cell responses.

PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273) are both B7 family members and are currently the only known ligands for PD-1 [3,7,8]. However, their effects are not exclusively mediated through PD-1 because PD-L1 interacts with B7-1, and PD-L2 can bind to another receptor, RGMb [9,10]. Although both PD-L1 and PD-L2 bind to PD-1 and deliver coinhibitory signals to T cells, their expression patterns differ significantly. PD-L2 is expressed in relatively few cells and tissues but is upregulated on activated antigen-presenting cells (APCs) including monocytes, macrophage, and DCs [4].

PD-L1 expression is much more diverse. PD-L1 can be seen on T cells, B cells, monocytes, macrophages, and DCs, and is typically upregulated with activation. Unlike the classic B7 family members, B7-1 and B7-2, which are mainly restricted to expression on APCs, PD-L1 is expressed in several non-hematopoietic tissues including the heart, pancreas, placenta, vascular endothelium, liver, lung, and skin [2,7]. This tissue expression plays an important role in regulating immune responses in the periphery [11,12]. In addition to these normal tissues, PD-L1 is often overexpressed on cancers as a mechanism for the cancerous cells to avoid immune surveillance. It is most likely that PD-L1/L2 expression on APCs and non-hematopoietic tissue (including tumors) is the most important from a therapeutic standpoint.

Glossary

Immune-related progression-free survival: in immune checkpoint inhibitor trials on initial treatment some lesions may progress or can worsen, which by traditional standards would have been considered progressive disease when it could actually be immune-mediated eradication of disease. Hence new criteria for the classification of immunological adverse events have been proposed and PFS measured according to the new criteria.
Immunological serious adverse events: adverse events which are autoimmune in nature and could have been potentially caused by immune drugs are termed 'immunological SAE' examples, autoimmune colitis, thyroiditis, pneumonitis.
Objective response rate (ORR): the proportion of patients with tumor size reduction of a predefined amount and for a minimum time-period.
Overall survival (OS): the percentage of people in a study who are alive for a specified period of time after they were diagnosed with or started treatment for cancer.
Progression-free survival (PFS): the PFS is defined as the time from assignment in a clinical trial until either progression of the disease or death of the patient due to any cause.
Serious adverse events (SAEs): unfavorable symptoms, signs, or laboratory values which, in the view of either the investigator or sponsor, result in any of the following outcomes: death, a life-threatening adverse event, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions (21CFR312.32). Adverse events are graded according to the Common Terminology Criteria for Adverse Events (CTCAE) on a scale of 1 to 5 where grade 1 is a mild adverse event and grade 5 is death. In clinical trials, grade 3 or 4 SAE usually require dose adjustment or stopping the drug.

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Beginning with the observation that PD-1 knockout mice develop spontaneous autoimmunity, it has since been demonstrated in numerous studies that the PD-1/PD-L1/L2 pathway is important for T cell regulation in a variety of infectious, autoimmune, and cancer models in mice [13]. These studies largely demonstrate an important role for these molecules in regulating T cell responses; this forms the basis for the development of a new generation of targeted therapies against PD-1 and PD-L1.

In this review we begin by covering the important roles of these molecules and their mechanisms of expression and signaling. This is an exciting time to review these molecules because we are only now beginning to see patients benefiting from over two decades of basic research focused on this pathway. We review the therapeutic potential of this pathway and summarize the latest clinical trial results of drugs targeting PD-1 and PD-L1.

PD-1 signaling

Signaling through PD-1 is triggered by engagement with its known ligands, PD-L1 and PD-L2. Despite the name of the receptor, cell death is not the primary result of engagement. Instead, the primary effect of this signaling is to inhibit TCR and essential costimulatory signals (Figure 1). Upon engagement, PD-1 clusters and localizes to the TCR complex [14]. PD-1 can inhibit the phosphorylation of the TCR CD3ζ chains and Zap-70, which are early steps following TCR engagement [14–16]. Downstream activation of Ras, an enhancer of survival and proliferation, is also inhibited by PD-1 [17]. Together with the direct TCR signals, CD28 delivers costimulatory signals by activation of the phosphatidylinositol 3-kinase (PI3K)/Akt pathway. PD-1 signaling represses this pathway by blocking PI3K activation [15]. This action begins with the phosphorylation of PD-1's intracellular immunoreceptor tyrosine-based switch motif (ITSM) and immunoreceptor tyrosine-based inhibitory motif (ITIM). The ITSM appears to be the more important of these two motifs [16,18]. The phosphorylated ITSM recruits the tyrosine phosphatase, SHP-2 [14,15]. This phosphatase leads to the inactivation of PI3K and downstream inhibition of the Akt pathway. Of note, although both PD-1 and CTLA-4 inhibit T cells, the mechanisms engaged by these two receptors are distinct [15].

The downstream signaling effects through PD-1 are numerous (Figure 1). As with other coinhibitory receptors, a decrease in T cell proliferation is seen together with a decrease in several inflammatory cytokines including

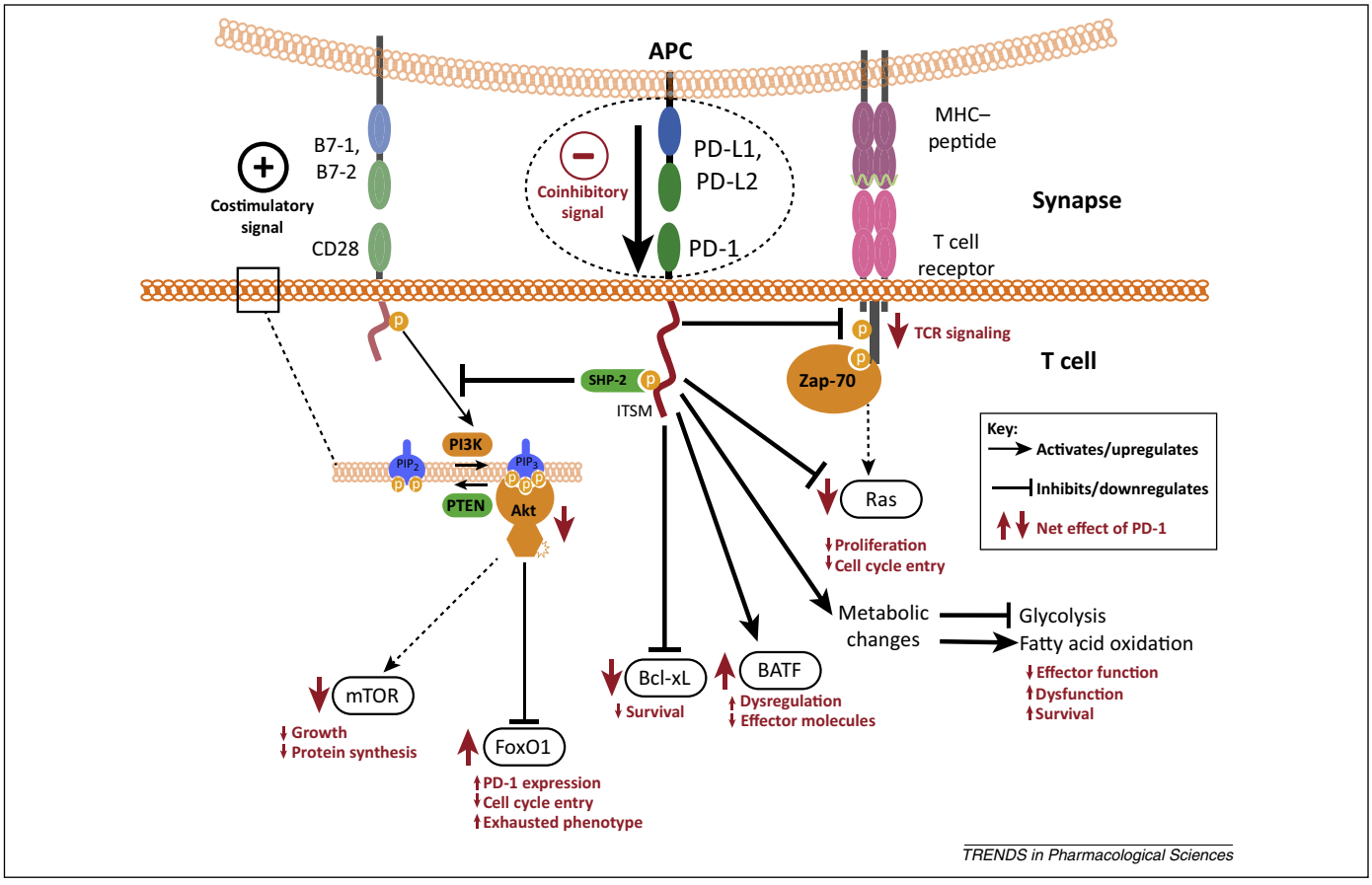

Figure 1. Programmed death-1 (PD-1) signaling. PD-1 has both an intracellular immunoreceptor tyrosine-based switch motif (ITSM) and immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic tail. SHP-2 can bind to the phosphorylated ITSM. Binding of ligands to PD-1 leads to overall inhibition of T cell receptor (TCR) signaling through inhibition of CD3ζ chain phosphorylation and Zap-70 association. PD-1 signaling causes the downregulation of both Ras and Bcl-xL which affect proliferation and cell survival, respectively. An increase in BATF can be seen which impairs the effector function of T cells. PD-1 also inhibits the phosphatidylinositol 3-kinase (PI3K)/Akt pathway by inhibiting the activation of PI3K. This has downstream effects including downregulation of mechanistic target of rapamycin (mTOR) and an increased half-life of FoxO1. PD-1 signaling also influences cellular metabolism by inhibiting glycolysis and promoting fatty acid oxidation. Together, all these effects cause T cells to become less proliferative, lose their effector functions, and take on an exhausted and dysfunctional phenotype. The net effect of PD-1 ligation on all of these processes is shown in red, with arrow direction indicating upregulation and downregulation. Abbreviation: APC, antigen-presenting cell.

tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), and interleukin 2 (IL-2) [2,3,6]. PD-1 signaling also appears to be self-reinforcing. Activation of this receptor protects the transcription factor, FoxO1, from degradation, which leads to increased expression of PD-1 [19].

More global effects are also seen on T cells. It has been shown that PD-L1 plays an important role in the differentiation of inducible regulatory T cells (iTregs) both *in vitro* and *in vivo* [20]. PD-L1 expression not only on APCs but also on other non-hematopoietic tissues may be capable of inducing Tregs. PD-1 signaling is accompanied by downregulation of phospho-Akt, mechanistic target of rapamycin (mTOR), S6, and Erk2, and by upregulation of phosphatase and tensin homolog (PTEN) [20]. Earlier work demonstrated that the Akt signaling pathway is a strong inhibitor of iTreg development, and this supports the proposed mechanism of the generation of PD-L1-induced Tregs [21].

It was also recently shown that PD-1 signaling influences the metabolism of T cells [22]. PD-1 signaling results in the inhibition of glycolysis and metabolism of amino acids while simultaneously promoting fatty acid oxidation [22]. These effects on T cell metabolism are consistent with an inhibition or reversal of effector function, and may partly explain the mechanism of impaired function seen in PD-1$^+$ T cells.

PD-1 plays an important role in exhausted T cells. It was first noted that, in chronic viral infections, PD-1 was upregulated selectively on exhausted CD8 T cells [6]. This observation has been seen in numerous chronic viral infections in both mice and humans [6,23–27]. PD-1 expression by T cells in the tumor microenvironment is also associated with an exhausted and dysfunctional phenotype [28]. Most importantly, blockade of the PD-1 signaling is able to restore CD8 T cell function and allows recovery of cytotoxic capabilities from the exhausted phenotype [29]. This treatment results in improved control of viral infection in several animal models and is the basis for future clinical trials manipulating PD-1 signaling in infectious disease.

Mechanisms controlling PD-1 expression

Considering the clinical importance of these molecules, there is great interest in understanding the mechanisms behind their expression. PD-1 is upregulated on T cells following TCR ligation [5] (Figure 2A). Cytokine signals are also important for the regulation of this molecule. Signaling through the common γ chain appears to be important, and ligands of the common γ chain, IL-2, IL-7, IL-15, and IL-21, can upregulate PD-1 expression on T cells [30].

Several more direct transcriptional mechanisms have been found as well. The transcription factor, T-bet, directly and actively represses *PD-1* gene expression [25]. After repeated antigenic stimulation, T-bet is downregulated, which leads to PD-1 expression and exhaustion. IL-6 and IL-12 (via STAT3 and STAT4, respectively) can also induce PD-1 in activated T cells through distal regulatory elements that interact with the *PD-1* gene promoter [31]. NFATc1 is a transcription factor that directly activates *PD-1* expression [31,32]. Blimp-1 inhibits *PD-1*

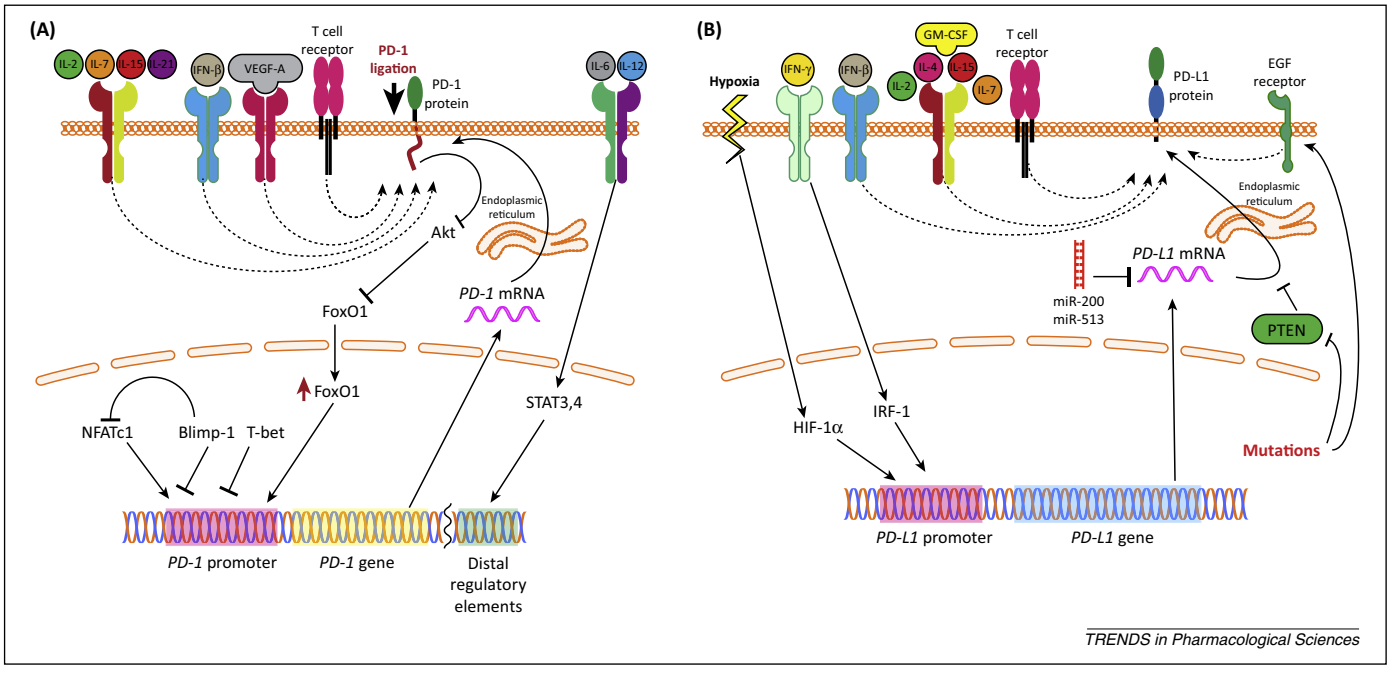

Figure 2. Regulation of programmed death-1 (PD-1) and programmed death ligand 1 (PD-L1) expression. PD-1 and its ligands are regulated by a complex network of factors. **(A)** PD-1 expression on T cells can be upregulated by numerous cytokines. Many of the common γ chain cytokines (interleukin-2, IL-7, IL-15, IL-21) can upregulate PD-1. IL-6 and IL-12, acting through signal transducer and activator of transcription 3 (STAT3) and STAT4, respectively, enhance expression of PD-1 through distal regulatory elements. Of particular relevance to the tumor microenvironment, vascular endothelial growth factor A (VEGF-A) can upregulate PD-1 through a VEGF receptor found on T cells. The nuclear factors FoxO1 and NFATc1 upregulate PD-1 through its promoter. Blimp-1 and T-bet also interact with the promoter but block its expression. Blimp-1 also functions by inhibiting NFATc1 promoter-binding. **(B)** PD-L1 expression is also regulated by numerous mechanisms. Like PD-1, several of the common γ chain cytokines upregulate it. IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) are also strong upregulators of both PD-L1 and PD-L2. In IFN-γ signaling, IRF-1 can bind to IFN response elements in the promoter of *PDL1*. Hypoxia can lead to upregulation of HIF-α which binds to the *PDL1* promoter and stimulates expression. Mutations of the EGFR receptor and loss of PTEN in tumors can upregulate PD-L1. Another post-transcriptional mechanism of regulation is through microRNAs. miR-200 suppression leads not only to cancer stage progression but also to simultaneous upregulation of PD-L1. miR-513 can similarly regulate PD-L1 expression in biliary epithelial cells.

expression in viral infection by not only repressing NFATc1 but also by generating suppressive chromatin changes at the *PD-1* locus [27]. Other epigenetic modifications have been described including regulation of *PD-1* by DNA methylation. Viral infection leads to a loss of this methylation in CD8 T cells which then allows transcription of *PD-1* [26,33]. This demethylation is directly related to the strength and duration of TCR signaling [26]. FoxO1 is another important transcription factor that promotes an exhausted cytotoxic T cell profile and upregulates *PD-1* [19]. FoxO1 is of particular importance because PD-1 signaling prevents FoxO1 degradation and thus defines a positive feedback loop where PD-1 signaling promotes the expression of more PD-1 [19].

PD-1 expression on T cells within the tumor microenvironment is a highly important factor in the use of immunotherapy for the treatment of cancers. PD-1 expression on T cells is predictive of response to therapies targeting this signaling pathway [34]. Beyond general T cell activation and local cytokines promoting expression of PD-1, it has been shown that vascular endothelial growth factor A (VEGF-A) can promote PD-1 expression on CD8 T cells through a VEGF receptor on these cells [35]. From all of these studies we can see that there is a complex network of many distinct mechanisms that influence the expression of PD-1.

Mechanisms regulating expression of PD-L1 and PD-L2

While PD-L1 and PD-L2 share some similarity in the molecules that induce them, there are some clear differences as well. Relatively little is known about the mechanisms regulating PD-L2 expression compared to PD-L1.

Several of the common γ chain cytokines, IL-2, IL-7, and IL-15, upregulate PD-L1 on monocytes and macrophages as well as on T cells (Figure 2B). IFN-γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-4 upregulate both PD-L1 and PD-L2 on macrophages [3,4]. IL-4 and GM-CSF appear to have the most profound effect on the expression of PD-L2. Downstream IFN-γ signaling specifically results in binding of interferon regulatory factor-1(IRF-1) to the *PD-L1* gene promoter [36].

PD-L1 overexpression on tumors has also been studied. While many of the mechanisms upregulating expression may be similar to those seen in leukocytes, several tumor-specific triggers have also been identified. Loss of PTEN is a common mutation in tumors and leads to overactivation of the PI3K/Akt pathway. This mutation and the ensuing downstream signaling can lead to overexpression of PD-L1 [37]. This overexpression mechanism is largely post-transcriptionally mediated. Similarly, there is evidence that overstimulation of the epidermal growth factor receptor (EGFR) pathway, which is often found in cancers with EGFR mutations, can lead to upregulation of PD-L1 in human cancer cells [38]. Another study showed a trend toward NRAS mutations being associated with higher PD-L1 levels [39]. Non-mutagenic mechanisms have also been established. It has been shown that several important signaling pathways including the PI3K and mitogen-activated protein kinase (MAPK) pathways can modify PD-L1 expression [40]. They also showed that pharmacologically manipulating these pathways may be a possible strategy to modify PD-L1 expression in tumors. Another group has shown specific evidence that treatment of melanoma patients with MAPK inhibitors will likely be beneficial in patients whose tumors express PD-L1 and contain tumor infiltrating lymphocytes (TILs) before treatment [41]. A feature common to nearly all solid tumors is hypoxia, which can lead to induction of the transcription factor, hypoxia-inducible factor-1α (HIF-1α). HIF-1α can bind to a hypoxia response element in the *PD-L1* promoter and lead to expression of PD-L1 not only on tumor cells but also on myeloid-derived suppressor cells (MDSCs), macrophages, and DCs within the tumor microenvironment [42]. MicroRNAs also play a role in regulating tumor-expressed PD-L1. Downregulation of miR-200 in tumors leads not only to metastasis but also a simultaneous enhancement of expression of PD-L1 [43]. In other tissues, miR-513 similarly targets degradation of the *PD-L1* transcript [44].

Immunotherapy targeting PD-1 in chronic infection

Chronic infection results in a sustained high level of antigen exposure, which ultimately leads to T cell exhaustion [45]. In a mouse model of chronic lymphocytic choriomeningitis virus (LCMV) infection, blocking PD-1 and lymphocyte-activation gene (LAG-3) simultaneously reversed the exhausted phenotype and led to the clearance of viral infection [46]. T cell exhaustion is also found in chronic infections such as HIV [47], and hepatitis B and C virus (HBV, HCV) infections in humans [48,49]. Reversal of the exhausted phenotype can be achieved by blocking PD-1, and this leads to clearance of the virus.

The proof of principle of this approach was demonstrated when the CTLA-4 inhibitor, tremelimumab, was tested in a Phase I trial in hepatocellular carcinoma and chronic HCV infection. Tremelimumab (15 mg/kg IV every 90 days) was administered until cancer progression. In this study, HCV viral loads declined in most patients and there was an increase in virus-specific IFN-γ-producing lymphocytes post-treatment [50]. Nivolumab, an anti-PD-1 monoclonal antibody, was tested in IFN-refractory (n = 42) and -naïve (n = 12) patients with chronic HCV infection [51]. Patients were randomized 5:1 to receive a single infusion of nivolumab in a dose-escalation protocol or of placebo (n = 7). Five patients in the nivolumab arm had a significant reduction in HCV RNA; three achieved a >4 log reduction, two patients achieved RNA below the lower limit of quantitation, and one remained RNA-undetectable 1 year post-study. Nivolumab was well tolerated and one patient had an asymptomatic alanine transaminase (ALT) elevation. Nivolumab and anti-PD-L1 treatments are being tested in HIV patients on antiretroviral therapy to eliminate the undetectable reservoir of viral infection. These studies show that reversing T cell exhaustion can be one strategy to control chronic viral infections.

Anti-PD-1 inhibitors in cancer therapy

The success of inhibiting the central immune check point, CTLA-4, in melanoma [52,53] led to the development of peripheral checkpoint inhibitors targeting the PD-1/PD-L1 pathway. PD-1 inhibitors block the interaction of the ligands, PD-L1 and PD-L2, with T cells and increase T cell proliferation and function [54]. The PD-1 inhibitors

currently in clinical trials are nivolumab (MDX-1106/BMS-936558, Bristol Meyers Squibb), pembrolizumab (MK-3475, Merck) and pidilizumab (CT-011, Cure Tech); these have some differences.

Nivolumab and pembrolizumab are fully human IgG4 and humanized IgG4 monoclonal antibodies (mAbs), respectively. Unlike the IgG1 and IgG3 subtypes, IgG4 has markedly decreased antibody dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) activity, which prevents depletion of activated T cells [55]. Below we present an overview of selected trials of PD-1 inhibitors in solid tumors and hematological malignancies (Table 1).

Melanoma

Melanoma is a known immunogenic tumor, and TILs in melanoma have been shown to colocalize with melanocytes expressing PD-L1. This interaction of T cells with tumor-expressed PD-L1 contributes to immune evasion in melanoma [56]. Ipilimumab (a monoclonal antibody against CTLA-4) demonstrated an overall survival (OS; see Glossary) benefit in two Phase III trials in metastatic melanoma [53,57]. However, approximately only 20% of patients with metastatic melanoma survive after 3 years even after ipilimumab, leaving marked room for improvement.

In a Phase I study of refractory melanoma patients, nivolumab had an objective response rate (ORR) of 31%

Table 1. Clinical trials of antibodies to PD-1[a]

Drug	NCT	Phase	Design	Population	*n*	Key findings/conclusions
Melanoma						
Nivolumab	NCT00730639	I	Dose finding and dose expansion	Advanced melanoma	107	OS = 43% at 2 years which compares favorably with historical population
	NCT01176461	I	Nivolumab +/- peptide vaccine (NY-ESO-1, gp100, MART-1)	Advance melanoma, Ipilimumab naïve and refractory	90	ORR = 25.1%. No difference in response between Ipilimumab naïve and refractory or addition of vaccine to nivolumab. PD-L1 negative patients also responded.
	NCT01721772	III	Nivolumab versus dacarbazine	Metastatic melanoma without BRAF mutation	418	Significantly better 1 yr OS in nivolumab arm (72.9% vs 42.1%). Relatively well tolerated
	NCT01721746	III	Nivolumab versus investigators choice	Metastatic melanoma after CTLA-4 or BRAF inhibitor therapy	370	Results in 167 patients showed higher ORR in nivolumab arm and durable tumor regression as well
Pembrolizumab	NCT01295827	I	Dose finding and dose expansion	Advanced melanoma including CTLA4 treated patients	135	ORR = 38%. No difference in response between ipilimumab naïve and refractory. Acceptable safety profile and slightly better responses in the higher dose 10 mg/kg arm
	NCT01295827	I	Nivolumab 2 mg/kg versus 10 mg/kg	Advanced melanoma whose disease progressed on ipilimumab	173	ORR = 26% after ipilimumab therapy. No difference between the two drug doses.
Pidilizumab	NCT01435369	II	Pidilizumab 1.5 versus 6 mg/kg	Advanced melanoma	103	Low response rate of 5.9%
Non-small cell lung cancer						
Nivolumab	NCT00730639	I	Dose finding and dose escalation	Advanced malignancies	296	ORR = 18.4% in NSCLC cohort. Low rate of Grade 3 SAE = 14%
	NCT01454102	I	Nivolumab + erlotinib	Stage IIB/IV NSCLC in EGFR mutated patients naïve or post progression	21	ORR = 19% with an acceptable safety profile
	NCT01642004	III	Open-label randomized nivolumab vs docetaxel	Metastatic squamous cell lung cancer after 1 line of platinum based therapy	272	Superior OS in the nivolumab arm at 1 year (9.2 vs 6 mos.) with durable responses
Pembrolizumab	NCT01295827	I	Pembrolizumab at 2 mg/kg or 10 mg/kg	Advanced NSCLC	282	ORR = 21%, PD-L1$^+$ tumors had higher response rates than negative tumors
Genitourinary malignancies						
Nivolumab	NCT01354431	II	Nivolumab at 0.3 versus 2 versus 10 mg/kg doses	Previously treated RCC with VEGF inhibitors	168	ORR = 21% across all 3 arms with a tolerable safety profile. No dose response relationship was response
Pembrolizumab	NCT01848834	Ib	Dose-finding study	PD-L1+ >1% and advanced urothelial cancer	33	ORR = 24%, with CR in 10% with an acceptable safety profile
Other cancers						
Nivolumab	NCT01592370	I	Dose escalation and dose expansion	Relapsed, refractory Hodgkin lymphoma	23	ORR = 87% with a PFS at 24 weeks of 86% with an acceptable safety profile.
Nivolumab	NCT01876511	II	Pembrolizumab 10 mg/kg q2 weeks	Advanced malignancies with or without mismatch deficiency	41	IrPFS = 78% versus 11% for mismatch deficient versus proficient colorectal cancers

[a]CR, Complete response; irPFS, immune related progression-free survival; NSCLC, non-small cell lung cancer; ORR, objective response rate; OS, overall survival, PFS, progression-free survival; SAE, serious adverse events.

with grade 3/4 serious adverse events (SAEs) in 22% of patients [58]. These results demonstrated both efficacy and acceptable safety of nivolumab in melanoma patients. In another Phase I study, prior treatment with ipilimumab or the addition of a peptide vaccine to melanoma antigens did not affect responses to nivolumab [59]. These results support basic research data showing that the immune checkpoints, CTLA-4 and PD-1, signal through mechanistically distinct pathways [15]. A randomized Phase III trial ($n = 418$) compared nivolumab at 3 mg/kg every 2 weeks ($n = 210$) with dacarbazine (chemotherapy) in BRAF-negative previously untreated metastatic melanoma [60]. The ORR (40% vs 13.9%), progression-free survival (PFS) (5.1 vs 2.2 months), and OS at 1 year (72.9% vs 42.1%) were significantly better in the nivolumab arm compared to dacarbazine. Moreover, grade 3/4 adverse effects were slightly reduced in the nivolumab arm (11.7 vs 17.6%) and immunological adverse events occurred in 1–2% of patients. In another open-label Phase III study, patients with metastatic melanoma who progressed on ipilimumab were randomized to nivolumab or to the investigators' choice of chemotherapy. The ORR was higher in the nivolumab arm (32% vs 11%), with durable tumor regression in responders [61]. Based on these results, nivolumab received FDA approval in December 2014 for patients with melanoma who were previously treated with ipilimumab or a BRAF inhibitor. Recently in a Phase I study the combination of two immune checkpoint inhibitors, ipilimumab and nivolumab, was safe and produced superior responses than ipilimumab alone for the upfront treatment of metastatic melanoma [62].

Pembrolizumab was studied in a dose-escalation study with a dose range of 1–10 mg/kg in 135 patients with refractory melanoma, some of whom received prior ipilimumab treatment [63]. The ORR was 38% and grade 3/4 adverse events were present in 13% of patients; there was no difference between ipilimumab-naïve and refractory patients. Based on these safety data KEYNOTE-001, an open-label trial, tested pembrolizumab in two doses at 2 mg/kg or 10 mg/kg after progression on ipilimumab and BRAF or MEK inhibitors, in BRAF-mutant tumors [64]. 173 patients with metastatic melanoma received pembrolizumab and the ORR was 26% in both groups and grade 3 to 4 SAEs were reported as 12%. The safety and efficacy of the 2 mg/kg and the 10 mg/kg doses were comparable with no significant benefit of the increased dose. Pembrolizumab was granted breakthrough status by the FDA for the treatment of ipilimumab- or BRAF inhibitor-refractory metastatic melanoma patients.

Non-small cell lung cancer

In non-small cell lung cancer (NSCLC), PD-1 is expressed in 35% of TILs and PD-L1 is expressed 20-25% of lung cancer specimens. Constitutive oncogenic signaling through the PI3K or EGFR pathway [37,38] or cytokine secretion by lymphocytes leads to activation of the PD-1/PD-L1 pathway in NSCLC [65].

Nivolumab was first tested in a dose-escalation Phase I trial of refractory malignancies of whom 129 had metastatic NSCLC [66,67]. The ORR in NSCLC was 18% with 33% of squamous and 12% of non-squamous cancers responding. The OS at 1 year was 42% and the median duration of response was 74 weeks and a sustained response of >24 weeks was seen in 57% of patients. Grade 3/4 toxicities were present in only 6% of patients and pneumonitis occurred in 7% of patients. Pneumonitis is a concern in these patients as they already can have poor lung reserve. Nivolumab was approved by the FDA for treatment of squamous NSCLC after progression on a platinum-based chemotherapy regimen. This approval was based on the results of an open-label, multicenter, randomized trial of 272 patients with metastatic squamous NSCLC who were randomized to docetaxel or nivolumab at 3 mg/kg every 2 weeks [68]. There was a significant improvement in median OS of 9.2 (nivolumab) versus 6 months (docetaxel) seen for patients receiving nivolumab. This represents a significant improvement for patients with squamous NSCLC whose treatment options are limited.

Pembrolizumab, in a pooled analysis of 262 relapsed NSCLC patients (KEYNOTE-001), had an ORR of 21% as a single agent, and results were similar in patients with squamous or non-squamous histology [69]. In patients with strong PD-L1 expression (>50%) the ORR was 39% and 16% in weak/negative expression suggesting that PD-L1 alone cannot be used as a biomarker to select patients. The FDA granted breakthrough status for pembrolizumab in lung cancer in October 2014.

Genitourinary malignancies

In renal cell cancer, increased TILs along with high PD-L1 expression in the initial biopsy is associated with shorter survival in patients treated with tyrosine kinase inhibitors (TKIs) for metastatic disease [70]. Similarly, high PD-L1 expression is associated with failure of response to Bacillus Calmette Guerin (BCG) for localized bladder cancer by neutralizing the T cell response to BCG immunotherapy [71]. These data suggest that the PD-1 axis contributes to resistant disease in urothelial malignancies.

Nivolumab was tested in a dose-escalation Phase I trial of patients with refractory malignancies of whom 33 had metastatic renal cell cancer with an ORR of 27% [66]. In a Phase II trial of 168 clear cell renal cell cancer (RCC) patients, nivolumab was tested at three doses of 0.3, 2, or 10 mg/kg and the median OS was 18.2, 25.5, and 24.7 months, respectively, which was higher than the historical OS rates of 11-16.5 months in this cancer [72]. As a result, a Phase III randomized study evaluating nivolumab and everolimus as a second-line therapy for metastatic RCC is underway.

Pembrolizumab was similarly tested in the KEYNOTE-012 study in 33 patients with metastatic urothelial cancer at 10 mg/kg and the ORR was 24.1% with a median OS of 9.3 months [73]. These studies show favorable efficacy and acceptable safety of PD-1 inhibitors in bladder and renal cancers and are highly likely to move forward in clinical trials.

Other tumors: Colon cancer, Hodgkin lymphoma

Hodgkin lymphoma is a B cell tumor in which the PD-1/PD-L1 axis is activated by JAK signaling and chromosomal amplifications in the 9p24.1 region which codes for the PD-L1/PD-L2 ligands. In an ongoing Phase I study of

Table 2. Clinical trials of antibodies to PD-L1[a]

Drug	NCT	Phase	Design	Population	*n*	Key findings/conclusions
Unselected						
BMS-936559	NCT00729664	I	Dose escalation and dose expansion	Advanced refractory malignancies	207	ORR: 6–17% and PFS: 12–41% at 24 weeks. Acceptable safety profile. Positive signal in melanoma, renal cell cancer, NSCLC and ovarian cancer with durable responses
MPDL3280A	NCT01375842	I	Dose escalation and dose expansion	Advanced solid tumor cancers	171	ORR = 21% with an acceptable safety profile. PD-L1$^+$ status resulted in higher responses. No pneumonitis related deaths
Genitourinary malignancies						
MPDL3280A	NCT01375842	I	Dose escalation and dose expansion	Metastatic urothelial bladder cancer	31	ORR = 50% with treatment response showing increase in CD8$^+$Ki67$^+$ T cells
Non-small cell lung cancer						
MEDI4736	NCT01693562	I	Dose escalation and dose expansion	Advanced NSCLC	13	ORR = 5/13 patients responded. No grade 3 pneumonitis observed

[a]CR, complete response; NSCLC, non-small cell lung cancer; ORR, objective response rate; OS, overall Survival; PFS, progression-free survival; SAE, serious adverse events.

23 patients with relapsed Hodgkin lymphoma, the ORR was 87% with a 17% complete response rate [74]. A recent Phase 2 study in colon cancer showed that immune-related progression-free survival rates were superior in mismatch-deficient compared to mismatch-proficient colon cancers (78 vs 11%) [46]. These studies show that these agents are likely to be effective across a wide variety of malignancies.

Anti-PD-L1 inhibitors in cancer therapy

Antibodies against PD-L1 act by blocking the interaction of PD-L1 with PD-1 but do not block the interaction of PD-1 with PD-L2. This may help to decrease toxicity since the PD-1/PD-L2 pathway still plays a role in peripheral tolerance. The three therapeutic monoclonal antibodies against PD-L1 are BMS-986559 (MDX-1105), MPDL3280A, and MEDI4736 and are in various phases of clinical trials. We briefly discuss here the clinical trials with these agents (Table 2).

BMS-936559 was first tested in a multicenter Phase I dose-escalation trial (0.3 to 10 mg/kg every 14 days in 6 week cycles) in patients with refractory malignancies [75], including melanoma, NSCLC, colorectal, renal cell, ovarian, pancreatic, and breast cancer (n = 207). The median duration of therapy was 12 weeks (range, 2 to 111) and SAEs occurred in 9% of patients. Patients with melanoma (9/52), renal cell (2/17), NSCLC (5/49), and ovarian cancer (1/17) had responses, and half of these responses were sustained for more than 1 year. It is currently not being developed as a clinical agent in malignancies despite its initial promise.

MPDL3280A is a bioengineered anti-PD-L1 antibody with minimal ADCC and CDC activity. In a Phase I dose-escalation trial of advanced solid tumors, no maximum tolerated dose (MTD) was defined at escalating doses [76]. The ORR was 21%, 24 week PFS was 44% and patients with PD-L1-positive tumors had a higher ORR (39%) than those with negative tumors (13%). Interestingly, there was no grade 3–5 pneumonitis or diarrhea in this small study, suggesting that the PD-L2 pathway (not inhibited by the anti-PD-L1 antibodies) could be important in minimizing toxicity. Further, in a Phase I study of urothelial cancer MPDL3280A showed significant activity (ORR 26%) with a good duration of response [77]. Based on these data, MPDL3208A received breakthrough status for bladder and NSCLC.

MEDI4736 is an IgG1 monoclonal antibody against PD-L1 that is being tested in an ongoing Phase I trial in NSCLC patients and shows preliminary clinical activity with a favorable toxicity profile [78]. Based on the Phase I results, a Phase III trial in patients with locally advanced NSCLC is being planned.

Concluding remarks

Under physiological conditions the PD-1 pathway is important for maintaining peripheral immune tolerance. This pathway represents one of the many redundant pathways to prevent inappropriate immune responses. Such redundant coinhibitory pathways are exploited by tumors and chronic viral infections to cause T cell exhaustion,

Box 1. Outstanding questions

Biomarkers of response

- Although therapies targeting PD-1 and PD-L1 are highly effective when they work, their current response rates leave much to be desired. Understanding what factors determine if a patient will respond is a crucial next step to advancing the use of these therapies. Predictors of response to immune checkpoint inhibitors could be related to tumor-associated factors or host factors. Tumor expression of PD-L1, specific mutations in the tumor, and the presence of tumor antigen-specific T cells are all examples of potential biomarkers currently being assessed. For example, PD-L1 expression will likely be a useful biomarker because patients with PD-L1 expression appear to have a higher response to anti-PD-1 therapy than those without [34]. In a retrospective analysis, NRAS mutant melanoma had a higher response rate to anti-PD-1 therapy [39]. CD8$^+$, PD-1$^+$, and PD-L1$^+$ cells in the tumor margins correlate with response to anti-PD-1 therapy in melanoma [34]. A highly restricted TCR repertoire also correlated positively with responses. In NSCLC, higher nonsynonymous mutational burden leading to increased neoantigens was associated with better responses to pembrolizumab [79]. Similarly, mismatch repair-deficient colon cancers which have a higher somatic mutational burden responded better to anti-PD-1 therapy [46]. In summary, there are clearly a variety of factors that control whether a patient will respond well to these therapies. Current and future work will address what these markers are and their relative importance. This work will be important not only for guiding therapeutic choices in patient treatment but also for finding strategies to enhance responses in patients treated with these drugs.

which results in tumor immune evasion and decreased viral clearance. Recent therapeutic advances targeting this pathway have met with good success in human cancers. More importantly, these treatments can provide durable responses. It remains to be seen whether combinatorial approaches with radiation, chemotherapy, other coinhibitory antibodies, or vaccines can improve the response rate in cancers. Predictive biomarkers need to be developed to identify short- and long-term responders to immunotherapy (Box 1). Different cancers may result in different mechanisms of PD-1/PD-L1 expression, and hence a single biomarker may not be useful across all tumor types. Tumor-related factors include specific oncogenic pathway activations, mutational burden, and PD-L1 expression, while host factors could be the presence of prior infections or vaccinations. Bioinformatics and immunogenetic approaches will be necessary to identify relevant tumor-associated antigens to which cytotoxic T cells respond or maintain response after immune checkpoint inhibitors.

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Review

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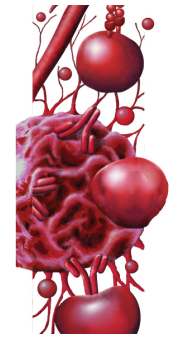
Immunotherapy



Emerging targets in cancer immunotherapy: beyond CTLA-4 and PD-1

Manipulation of co-stimulatory or co-inhibitory checkpoint proteins allows for the reversal of tumor-induced T-cell anergy observed in cancer. The field has gained credence given success with CTLA-4 and PD-1 inhibitors. These molecules include immunoglobulin family members and the B7 subfamily as well as the TNF receptor family members. PD-L1 inhibitors and LAG-3 inhibitors have progressed through clinical trials. Other B7 family members have shown promise in preclinical models. TNFR superfamily members have shown variable success in preclinical and clinical studies. As clinical investigation in tumor immunology gains momentum, the next stage becomes learning how to combine checkpoint inhibitors and agonists with each other as well as with traditional chemotherapeutic agents.

Keywords: B7 family • checkpoint proteins • immunotherapy • TNFR superfamily • translational medicine

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One of the hallmarks of cancer is the ability of the malignant cell to escape eradication by the immune system [1]. Proposed over a century ago, the concept of immune control of cancer continues to develop [2,3]. The existence of tumor antigens led Burnett and Thomas to form their hypothesis about cancer immune surveillance where the adaptive immune system was responsible for preventing the development of cancer in immunocompetent hosts. This hypothesis fell out of favor until the 1990s when improved mouse models of immunodeficiency were developed and particularly when the role of IFN-γ in promoting immune-mediated rejection of transplanted tumor in mice [4].

Tumors are variably infiltrated by cytotoxic T lymphocytes (CTLs), but a dense infiltration portends a better prognosis [5–7]. The T-cell response follows a complex interaction between an antigen-presenting cell (APC) and a T cell. TCR recognition of an antigen on MHC molecule is not sufficient, a second signal provided by a member of the B7 family is required [8]. CD28 provides the primary co-stimulatory signal for the activation of T cells after it engages B7-1 (CD80) or B7-2 (CD86) [9]. CTLA-4 is a CD28 homologue that interacts with B7-1 and B7-2 and, in contrast to CD28, provides an inhibitory signal [10,11]. Newly identified members of the B7 family also provide inhibitory signals the roles of which continue to be explored [12]. Blocking CTLA-4 mediated inhibition of the T-cell effector response has been an attractive therapeutic target. Monoclonal antibodies (mAb) that block CTLA-4 are effective in mouse models of a variety of tumors [13–15]. Ipilumumab (Yervoy®) is US FDA approved for the treatment of metastatic malignant melanoma and represents the first success story of T-cell checkpoint inhibitor immunotherapy [16].

A more recent success story in cancer immunology is that of PD-1. PD-1 was first identified in lymphoid cells lines induced to undergo programmed cell death [17]. Later reports noted that PD-1 is expressed on activated T and B cells, dendritic cells (DCs) and monocytes upon stimulation where it is found to play an inhibitory role [18–20]. PD-1 is highly expressed on T cells and leads to

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T cell exhaustion [21,22]. PD-1 expression is also noted on $CD4^+$ $Foxp3^+$ regulatory T cells (Tregs) where it contributes to their inhibitory role [23]. Several mAbs targeting PD-1 have progressed through clinical trials. The first FDA approved mAb was pembrolizumab (Keytruda®), also known as lambrolizumab after showing response rates in melanoma patients who have progressed after first line therapy including immunotherapy with ipilimumab (KEYNOTE-001 trial) or in comparison to investigator-choice chemotherapy (KEYNOTE-002) [24,25]. The second PD-1 targeting mAb to receive FDA approval was nivolumab (Opdivo®). It was well tolerated in Phase I studies in solid tumors as well as lymphomas [26–30]. Similar to pembrolizumab, nivolumab was shown to be superior to chemotherapy in the second line setting in melanoma in the Phase III CheckMate-037 trial [31,32]. Nivolumab yielded better survival and higher response rated in comparison to docetaxel in the treatment of advanced squamous non-small-cell lung cancer [33]. Other PD-1 mAbs include pidilizumab (CT-011) which was the first one to reach clinical trials and remains in development (studies reviewed in [34]) and more recently MEDI0680 which is entering clinical trials [35,36].

Blocking CTLA-4 and PD-1 are not the exclusive path toward T-cell 'dis-inhibition'. A variety of immunomodulatory pathways have been studied and exploited clinically with varying degrees of success and are at different stages of clinical development. Other members of the B7 family, part of the immunoglobulin superfamily, include B7x, HHLA2 and B7-H3 which play an inhibitory role. VISTA, Tim-3 and LAG-3 are members of the immunoglobulin superfamily have also been shown to play an inhibitory role. Immunomodulatory pathways include members of the TNF receptor family and their ligands which have been studied as targets for cancer immunotherapy. These inhibitory and stimulatory molecules that have been studied as therapeutic targets are depicted in Figure 1A & B, respectively. Finally, indoleamine 2,3–dioxygenase 1 inhibitors have also been studied as antitumor therapies as discussed below.

PD-L1 & PD-L2

The first reported ligand for PD-1 is PD-L1 (B7-H1) with wide expression at the mRNA level in lymphoid and nonlymphoid tissues [37]. It is a cell surface protein that is expressed on activated APC, T and B lymphocytes and other cells. It inhibits TCR mediated T-cell proliferation and cytokine production through the engagement of PD-1 [38]. The PD-1/PD-L1 interaction induce T-cell tolerance in lymphoid tissue before their exit to the periphery, and blockade of this interaction can reverse T-cell anergy [39]. Additionally, PD-L1 expressed on tumor cells can also act as a ligand to deliver an anti-apoptotic signal that leads to resistance to cytolytic function of CTL as well as to Fas-induced and drug-induced apoptosis [40]. Another interesting fact is that B7-1 was also shown to interact with PD-L1 which results in inhibition of T cells [41,42]. A second ligand for PD-1 is PD-L2 (B7-DC), which inhibits TCR mediated T-cell proliferation and cytokine production [43,44]. It is mainly expressed on DCs and macrophages [44,45]. Recently, a novel binding partner for PD-L2 is identified named RGMb, which is important for the development of respiratory immune tolerance [46].

PD-L1 is expressed in a variety of human carcinoma specimens as well as hematological malignancies such as multiple myeloma, leukemia and peripheral T-cell lymphoma and has been correlated to poor prognosis [8,34]. Several mAbs that target PD-L1 have reached clinical trials. BMS–936559 is a fully human monoclonal IgG4 antibody that blocks PD-L1 [47]. Anti-PD-L1 antibodies inhibited tumor growth in murine syngeneic tumor models with a durable antitumor immunity. BMS–936559 can reverse *in vitro* Treg mediated suppression and does not cause antibody-dependent cytotoxicity or complement-dependent cytotoxicity [47]. The first clinical trial with BMS–936559 also demonstrated high tolerability and durable responses [47]. Other monoclonal anti-PD-L1 antibodies include MEDI4736 [48,49], atezolizumab (MPDL3280A) which demonstrated a 43% response rate in a Phase I clinical trial in metastatic urothelial bladder cancer patients resulting in an FDA breakthrough designation [50], and MSB0010718C which exhibits antitumor activity by blocking PD-L1 as well as antibody-dependent cell-mediated cytotoxicity [51,52].

B7x (B7-H4/B7- S1)

B7x is an inhibitory transmembrane protein that binds activated T cells and is a member of the B7 family [53–55]. It inhibits CD4 and CD8 cell proliferation and cytokine production [53]. It is hardly expressed on professional APC but is expressed on nonlymphoid tissues, mainly epithelial tissues where a role in immune tolerance is postulated [54,56–58]. It is expressed in the lung epithelium and is implicated in attenuating the immune response to bacterial infection in mice [56]. B7x is expressed in a variety of human cancers which include cancers of the brain, esophagus, lung, breast, pancreas, kidney, gut, skin, ovary and prostate [59]. Prostate cancer specimens from patients treated with radical prostatectomy had 15% prevalence of B7x expression and high expression was significantly associated with a higher risk of prostate cancer related death [60]. B7x expression in renal cell carcinoma is

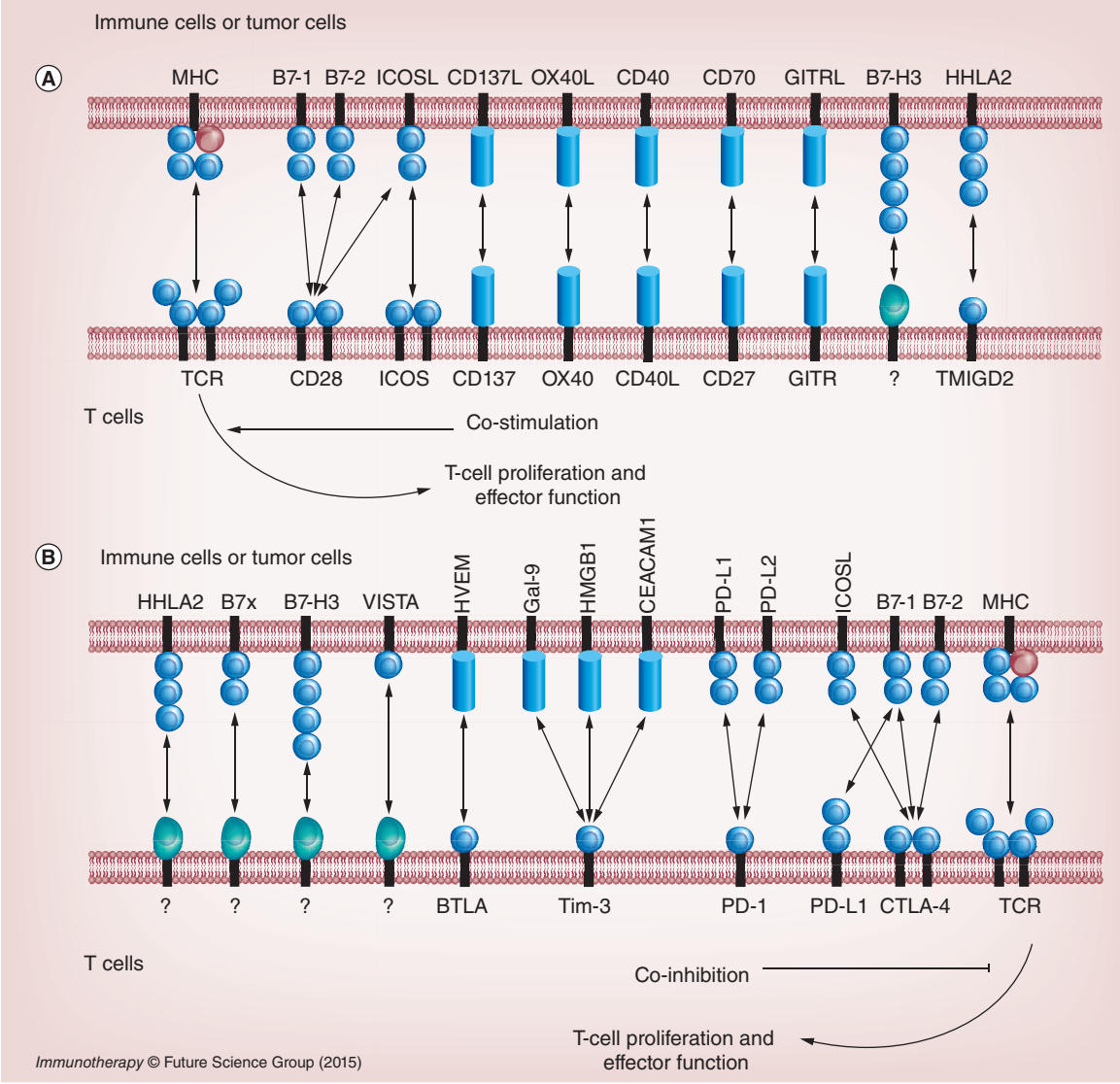

Figure 1. Summary representation of T-cell molecules. (A) Summary representation of T-cell co-stimulatory molecules. **(B)** Summary representation of T-cell co-inhibitory molecules.

associated with adverse clinical and pathological features as well as poor survival [61]. Tumor expression of B7x in human gastric cancer predicts poor survival [62]. Similar findings were also reported in studies of ovarian cancer and lung cancer [63,64]. In a preclinical model, mouse colon carcinoma cells line CT26 transfected with murine or human B7x resulted in a higher number of lung metastasis and shorter survival [65]. Blockade of B7x with a mAb resulted in a reduction of number of lung metastasis in a CT26 as well as 4T1 based mouse models of lung metastasis [65]. B7x thus represents a very promising target for cancer immunotherapy.

HHLA2 (B7y/B7-H5/B7H7)

HHLA2 is another member of the B7 family that modulates T-cell function [66,67]. It is expressed on monocytes and induced on CD19 positive B cells. HHLA2–Ig fusion protein bound resting and activated CD4 and CD8 T cells, as well as APC. It was shown to inhibit proliferation of CD4 and CD8 T cells in the presence of TCR signaling as well as T-cell cytokine production [66]. TMIGD2, also called CD28H or IGPR–1, is identified as one of the receptors for HHLA2 [67,68]. IGPR–1 was initially reported to be an adhesion molecule involved in angiogenesis [68]. HHLA2 expression in non-lymphoid tissues was limited to placenta, GI tract, kidney, gallbladder and breast, but its expression was more common in human tumor specimens including breast, lung, thyroid, melanoma, pancreas, ovary, liver, bladder, colon, prostate, kidney and esophagus [68]. In a cohort of 50 patients with triple negative breast cancer, 56% of patients had HHLA2 expression

on their tumors, and high HHLA2 expression was significantly associated with regional lymph node metastasis and stage. Of interest, increase in HHLA2 expression was also due to an increase in gene copy number, and not just stimulation [68]. There is much to be discovered about HHLA2 and it represents a potential target for cancer immunotherapy.

B7-H3

B7-H3 was first identified as a molecule that binds a receptor on activated T lymphocytes [69]. Its expression was inducible on DCs and was initially thought to be costimulatory to T lymphocytes [69]. *In vivo* studies in mouse models showed that B7-H3 was an inhibitory to T lymphocytes and preferentially inhibits T helper cells type 1 response [70]. The receptor for this ligand is still unclear. It is expressed in some cancer cells and was associated with regional nodal metastasis [12,71]. Currently the majority of evidence suggests that this is a co-inhibitory ligand for T-cell response [72]. B7-H3 was found to be upregulated in graft-versus-host disease (GVHD) target organs and its absence in B7-H3$^{-/-}$ mice resulted in augmented GVHD lethality and T-cell proliferation and function [73]. Increased B7-H3 expression in cancer specimens has been reported [74], and has been correlated to worse outcomes [60,75]. Therefore, B7-H3 is another potential target for cancer immunotherapy.

VISTA

VISTA is a recently discovered negative modulator of the immune system [76]. VISTA is primarily expressed on hematopoietic cells, including APCs and T cells [77]. It is a suppressor of CD4 and CD8 T cells. In addition, within the CD4 subset, both effector and memory T-cells are effectively suppressed. T cells cultured with soluble VISTA-Ig fusion protein show no shift in expression of CD45RA to CD45RO [77]. Another notable phenomenon is that, while it blocks proliferation, it does not induce apoptosis and thus the cells remain viable. Lastly, the suppressive effect on T cells appears to be long lasting, even after VISTA effect was removed. In addition to being immunosuppressive of T cells, it is also immuneregulatory. Under neutral conditions, VISTA-Ig is capable of suppressing naïve T-cells from forming Treg. This however was not the case when the culture conditions were changed, and in the presence of IL-2 and anti-CD28 VISTA-Ig was actually able to increase the proportion of Treg. Of note, VISTA does not appear to have any effect *in vitro* on B-cell proliferation or regulation. Its effect on cytokine production included reduced levels of IL-10, IFN-γ and TNF-α [77]. Anti-VISTA mAb exacerbate experimental auto-immune encephalomyelitis as well as enhancing antitumor immune responses [76]. Anti-VISTA mAbs are able to increase the number of tumor specific T cells in the periphery and enhance the infiltration, proliferation and effector function of tumor infiltrating lymphocytes within the tumor microenvironment [76]. In a melanoma model, both transplantable and inducible cancers are suppressed effectively with anti-VISTA monotherapy [78]. VISTA's antitumor effect was also explored in concert with a peptide-based cancer vaccine where VISTA blockade synergistically impaired tumor growth.

CD27

CD27 is a T-cell differentiation antigen and member of the TNFR superfamily [79]. It has increased membrane expression on anti-CD3-activated T cells. Agonistic CD27 mAb resulted in enhanced proliferation of CD3 stimulated T cells. CD27 and its ligand CD70 are thought to have important effects on T-cell function [79]. Using intranasal influenza virus infection as a model system, CD27 has been shown to be a major determinant of CD8 T-cell priming at the site of infection. CD27 signaling, along with other signaling including CD28, is crucial for the generation of antigen specific CD8 T cells. Via cell survival stimulation, CD27 promotes accumulation of activated T cells thereby expanding the proportion of virus specific T cells [79]. The survival signal relies on IL-2R signaling and autocrine IL-2 production and CD27 is responsible for long-term survival of primed CD8 T cells, and hence memory. CD27 function has been extensively studied in mice and is transiently expressed during the germinal center reaction. CD27 expression is most abundant during the phase of expansion of primed B cells and is absent from memory B cells. It appears that CD27 T cells provide help to B cells to form small germinal centers. Additionally, CD27 signaling in B cells results in enhanced levels of plasma cell formation and increased IgG production. Interestingly, constitutive CD27 signaling could have alternate effects on cellular and humoral immunity [79]. Collectively, the data elucidated CD27 signaling as a determinant of germinal center kinetics. In mouse models, costimulatory effect of CD27 was necessary for anti-CD40 antitumor efficacy [80]. Antitumor efficacy was shown in a mouse model of lymphoma using an anti-CD27 mAb. Anti-CD27 demonstrated no effect in SCID mice suggesting the need for an intact adaptive immune response and that the response itself was not due to a direct effect on the lymphoma cells [80].

A fully human anti-CD27 mAb, IF5, increased survival in a mouse model of leukemia and lymphoma [81]. Its toxicity was assessed in a non-human primate model and has entered clinical development under the name

CDX–1127 (Varlilumab) and was assessed in a Phase I clinical trial [82]. The drug was well tolerated and responses included a complete response in a patient with stage IV Hodgkin's lymphoma who had previously failed stem cell transplant, chemotherapy and brentuximab-vedotin and three additional patients with stable disease.

OX40/OX40L

OX40 is a member of the TNFR superfamily and is expressed on activated CD4 and CD8 T cells as well as other lymphoid and nonlymphoid cells [83]. It is also expressed in natural killer (NK) cells, NKT cells and neutrophils. OX40L is also a member of the TNFR superfamily and its expression is inducible on APC. Nonlymphoid cells can also be induced to express OX40L which supports of the role of the OX40/OX40L pathway in regulating the T-cell response. T cells themselves can express OX40L which represents an additional mechanism for T-cell response amplification. TCR signaling is sufficient to induce OX40 in activated CD4 and CD8 T cells, however this is augmented by CD28 and B7-1/B7- 2 interaction and modulated by cytokines such as IL-1, IL-2 and TNF [83]. OX40L expression on the other hand is can be induced on APC upon activation by ligation of CD40 or by Toll-like receptors [83].

The OX40/OX40L pathway plays a large role in T cell expansion and survival, primarily by maintaining later proliferation and T-cell survival through the effector phase [83]. OX40 ligation can also directly inhibit naturally occurring Treg activity in mice providing another means to promoting effector T-cell proliferation and survival [83]. Foxp3 expression on naïve CD4 T cells is blocked by OX40/OX40L activity which supports a role in the suppression of naïve CD4 T-cells differentiation to become Treg. There have been conflicting reports however on the impact of OX40 signaling, which is expressed constitutively expressed on Treg, and may in fact promote Treg responses depending on the cytokine milieu [84].

The rationale for targeting OX40/OX40L signaling for cancer immunotherapy is supported by the expression of OX40 in tumor infiltrating lymphocytes. Preclinical models have shown that injection of agonist OX40L-Ig fusion proteins, OX40 mAb, RNA aptamers that bind OX40 and transfection of tumor cells or DCs with OX40L can all suppress tumor growth [83]. In mouse models of cancer including sarcoma, melanoma and glioma, among others, OX40 activity has been shown to decrease tumor growth [84]. The mechanism of tumor growth suppression is related to CD8 T-cell survival, and/or promotion of CD4 T-cell help for CD8 T cells. There may be an additional role via augmentation of NK cell activity. Tumor infiltrating Tregs express high levels of OX40, and the signaling of OX40 within this environment suppressed their activity [85]. In animal models, stimulation of OX40 appears to both augment antitumor activity as well as suppress Treg activity. Human studies include a Phase I trial using anti-OX40 mab 9B12 in patients with advanced cancers (NCT01644968 [86]) [87]. Therapy was well tolerated with no maximum tolerated dose reached. Most common grade 3 or 4 side effects included lymphopenia that was transient. Best response to therapy by response evaluation criteria in solid tumors (RECIST) only included stable disease with some tumor regression noted but less than 30% of overall tumor. One limitation of this agent was the induction of human antimouse antibodies, which precluded patients from receiving additional cycles. Nevertheless, this study provides evidence in humans that OX40 agonism can augment the immune system by stimulating CD4 and CD8 T-cell proliferation, CD8 IFN-γ production and increased antibody titers and T cell recall in response to tetanus immunization.

It seems that targeting OX40 alone may not be sufficient to elicit a robust antitumor response, and thus combination immunotherapy, particularly with antagonistic anti-CTLA-4 and anti-PD-1 antibodies, has been an area of study in preclinical models [84]. Combination therapies may stimulate complimentary pathways that synergistically respond to poorly immunogenic or large tumors, which has been an area of weakness in immunotherapy. Naturally, synergism may also extend to worsening the toxicity profile of such therapy but the fact the anti-OX40 therapy was fairly well tolerated may be promising.

CD40/CD40L

CD40 and its ligand CD40L are members of the TNFR/TNF family [88]. CD40 is expressed on professional APC as well as other non-immune cells and tumors. Its ligand, CD40L is transiently expressed on T cells and other non-immune cells under inflammatory conditions [88]. Inherited lack of CD40L is responsible for x-linked hyper-IgM syndrome (H-XIM). It activates DCs and allows them to stimulate CD8 T-cell activation and proliferation [89].

Binding of CD40L to CD40 promotes CD40 clustering on the cell surface, as well as recruitment of adapter proteins known as TRAFs to the cytoplasmic domain of CD40 [88]. CD40 signaling is carried forward by different pathways which include MAPKs, NF kappa B, PLC and PI3K. Additionally, it is noted that JAK3 binds the cytoplasmic domain of CD40 and can mediate other cellular processes. CD40/CD40L can directly activate DCs [89]. This CD40/CD40L inter-

action is necessary for the maturation and survival of DCs and CD40-dependent maturation of DCs leads to sustained expansion and differentiation of antigen specific T cells. The increased life span of DCs is very important in driving cell-mediated immunity. Without the CD40 survival signal, passive apoptosis of T cell is induced [88].

CD40 signaling by B cells is required for the generation of high titers of isotype switched high affinity antibody as well as for the development of humoral immune memory [88]. The binding of CD40L on CD4 T cells to CD40 on activated B cells is an important step in initiation and progression of the humoral immune response. Once CD40 signaling is active, there are downstream effects including B-cell intracellular adhesion, sustained proliferation, differentiation and antibody isotype switching. This process is essential for memory B cells and long lived plasma cells. B-cell fate is heavily influenced by CD40 signaling [88]. This signaling via binding of CD40 and CD40L can help determine whether the maturing B cell becomes a plasmablast or seeds a germinal center. With the activated CD40 pathway, the B cell will go on to form a germinal center. The lack of CD40 signaling is sufficient to block germinal center formation. T helper cells are recruited to these germinal centers, some of which express CD40L on their cell surface, which serve to maintain the germinal center. The lack of CD40 signaling in germinal center B cells increase Fas dependent apoptosis.

CD40 is expressed in mouse and human models of melanoma, prostate and lung cancers, and carcinomas of the nasopharynx, bladder cervix and ovary [88]. Hematologic malignancies such as non-Hodgkin's lymphoma, Hodgkin's lymphoma, acute leukemias and multiple myeloma also express CD40. Anti-CD40L mAb treatment inhibits the generation of protective immune responses from potent tumor vaccines [90]. Additionally, using CD40 deficient mice, no protective antitumor immune response was induced following a protective vaccination regime. Early studies using a lymphoma model showed that agonistic anti-CD40 antibodies were able to eradicate tumor. Gene delivery of CD40L to DCs and tumor cells was sufficient to stimulate a long lasting systemic antitumor immune response in a murine model [90]. The approach of gene therapy with adenovirus expressing CD40L has been shown to be successful in colorectal carcinoma, lung carcinoma and melanoma murine models. CD40 agonism alone, however, was not sufficient for antitumor response likely due to the lack of TLR signaling.

Clinically, recombinant CD40L has been used in patients with solid tumors or non-Hodgkin's lymphoma given subcutaneously daily for 5 days in a Phase I clinical trial [91]. Responses to therapy included 6% of patients with a partial response and one patient with a complete response. Humanized agonistic CD40 mAb include CP–870,893, SGN-40 and HCD 122. In a Phase I trial, CP-870,893 produced a partial response in 14% of patients (27% of melanoma patients). Dose-limiting toxicities were observed and included cytokine release syndrome [88]. Dacetuzumab (SGN-40) is a weak agonist and was studied in a Phase I trial in patients with refractory or recurrent B-cell non-Hodgkin's lymphoma [92]. Toxicity was shown to be acceptable and antitumor activity was seen with six objective responses, 13 patients with stable disease. The overall response rate for patients in this cohort was 18%. In a Phase II trial with this drug, 46 patients again with relapsed or refractory diffuse large B-cell lymphoma were treated and the overall response rate was 9% and disease control rate (stable disease or better) was 37% [92]. Lucatumumab (HCD122) is a fully human anti-CD40 antagonist and was tested in 28 patients with refractory or relapsed MM [93]. Responses included 12 patients with stable disease, and one patient maintaining a partial response for greater than 8 months. It was also well tolerated with a good safety profile.

CD137(4-1BB)/CD137L

CD137, also known as 4-1BB, is an induced T-cell costimulator molecule and a member of TNFR superfamily [94]. CD137 is induced on activated CD4 and CD8 T cells, NK cells and constitutively on DCs, Tregs, monocytes and myeloid cells [94,95]. Its ligand 4-1BBL is expressed on B cells, DCs, macrophages, activated T cells and endothelial cells [94–96]. Agonistic CD137 mAb stimulated the proliferation of CD4 and CD8 T cells, mainly CD8 CTL, increased cytokine production, prevent activation induced cell death [94,97] and in absence of cognate signals increases the memory T-cell expansion [98]. Large tumors in mice are eradicated with increased cytotoxic T-cell activity in poorly immunogenic Ag104A sarcoma and highly tumorigenic P815 mastocytoma using agonist CD137 mAb [99]. CD 137 agonist mAbs also increase adhesion molecules ICAM-1, VCAM 1 and E-selectin thus increasing the trafficking of activated T-cells into tumor and also prevent immune tolerance by preventing induction of CD8 CTL anergy to soluble tumor antigens [100].

Urelumab (BMS 663513) is humanized IgG4 mAb and PF 05082566 is humanized IgG2 mAbs for CD137 which are currently in clinical development [101,102]. BMS 663513 was tested in a Phase I/II trial with locally advanced and metastatic solid tumors. Initially patients with melanoma have been

enrolled but enrollment has been expanded to include renal cell and ovarian cancer patients [103]. Treatment is well tolerated and responses included three partial responses and four with stable disease [104]. A randomized Phase II trial (NCT00612664) in metastatic melanoma patients as a second-line therapy was terminated due to grade 4 hepatitis [95]. Urelumab is being tested with rituximab in B-cell non-Hodgkin's lymphoma or chronic lymphocytic leukemia as enhanced antibody-dependent cytotoxicity by rituximab was noted after activation of NK cells with CD137 [105]. Addition of anti-CD137 mAb to cetuximab improves efficiency of cetuximab in head and neck tumors as well as *KRAS* mutant and wild-type colorectal cancer, which provides further evidence for the use of immunotherapy, specifically anti-CD137, in combination with other agents [106]. PF–05082566 was tested in a Phase I study of 27 patients and is well tolerated with mostly grade 1 adverse effects with one grade 3 elevated alkaline phosphatase was noted [107].

GITR

GITR is a member of TNFR superfamily and is a co-stimulatory receptor. It has been originally discovered as up regulated in dexamethasone treated murine T-cell hybridomas [108]. It has very low expression in human T cells but constitutively expressed in human Treg [109]. Upon stimulation, naïve T cells and Treg upregulate GITR in a similar fashion to 4-1BB and OX40 suggesting their role in latter time points rather than early priming [110]. GITR-L is expressed in DCs, macrophages and B cells and is upregulated upon activation. GITR-L is also found in endothelial and activated T cells and may have role in leukocyte adhesion [110]. Its function is similar to OX40 and 4-1BB; it sends costimulatory signals inducing T-cell proliferation, effector function and protects T cells from activation induced cell death [110]. Combined anti-PD-1 blockage and GITR costimulation has potent antitumor activity in murine ID8 ovarian cancer model and is synergistic with chemotherapeutic agents. This combination promotes accumulation of CD4, CD8 T cells with decreased Treg and myeloid derived suppressive cells [111]. TRX518, an anti-GITR mAb, is being studied in a Phase I study in patients with stage III or stage IV melanoma or other solid tumor malignancies (NCT01239134 [86]). To circumvent autoimmune complications from immunomodulators mRNA transfected DCs are used locally to deliver anti CTLA-4 mAb and soluble GITR-L while increasing the antitumor immune responses [112]. Phase I clinical trial of a DC vaccine that entails intranodal injection of DCs transfected with mRNA encoding tumor antigens along with DCs transfected with mRNA encoding soluble human GIRT-L and anti CTLA-4 mAb is in progress (NCT 01216436 [86]).

Tim-3

Tim-3 was first described in 2002 [113]. It is expressed on CD4 T cells and CD8 CTLs. Tim-3 binds several molecules including Gal-9, CEACAM1, HMGB1 as well as glycosylated molecules [114]. Binding of its ligand, Gal-9 induces cell death and thus illustrating its role as a negative regulatory molecule, with particular importance in Th1- and Tc1-driven responses. Tim-3 is expressed on all IFN-γ secreting Th1 cells as well as DCs [113]. Th1 immunity is regulated via binding of its ligand, Gal-9, directly triggering cell death. It has also been shown that Tim-3/Gal-9 binding also suppresses immune responses indirectly by expanding the population of myeloid derived suppressor cells [113]. Tim-3 has been implicated in inducing T-cell exhaustion in several scenarios including chronic viral infections such as hepatitis C and HIV, bacterial infections and cancer [113]. In murine models of colon adenocarcinoma, melanoma and mammary adenocarcinoma, Tim-3 can be co-expressed with PD-1 in tumor infiltrating CD4 and CD8 T cells. Tim-3^{+}PD-1^{+} CD8 T cells were among the most impaired T cells with reduced proliferation and decreased production of IL-2, TNF and IFN-γ. *In vivo*, Tim-3 blockade in concert with PD-1 blockade produced a significantly higher antitumor effect than either one alone. Additionally, this combined blockade increased the frequency of proliferating antigen specific CD8 T cells.

LAG-3

LAG-3, also called CD223, is expressed on activated T cells, NK cells, B cells and tumor infiltrating lymphocytes [115]. It is closely related to CD4; it is a member of the immunoglobulin superfamily and its gene is located near CD4 on chromosome 12. LAG-3 is a negative regulator of T-cell activation and homeostasis [115]. LAG-3 binds to MHC class II molecules with high affinity [116]. LAG-3 cross-linking on activated human T cells induces T-cell functional unresponsiveness and inhibits TCR-induced calcium ion fluxes. Similar to CD4 and CD8, LAG-3 is considered to be a coreceptor to the CD3–TCR complex. Inhibition of cytokines, such as IL-2, is induced by LAG-3 *in vitro*. It decreases the pool of memory CD4 and CD8 T cells. It also increases the suppressive activity of Treg. For maximal suppressive activity of Treg, LAG-3 signaling is required [117]. It was not clear however, if the signal alone was sufficient. Within the tumor microenvironment LAG-3 promotes immune tolerance of the tumor by inhibiting APC and T-cell function [118].

Malignant mouse and human tissue has been shown to co-express PD-L1 and LAG-3 [119]. A significant percentage of tumor infiltrating CD4 and CD8 T cells from mouse tumor models of melanoma, colorectal adenocarcinoma and fibrosarcoma, express high levels of LAG-3 and PD-1. When using anti-LAG-3 immunotherapy, reduced growth of fibrosarcoma and colorectal adenocarcinoma is observed in some mice. This same effect is seen with anti-PD-1 monotherapy. Anti-LAG-3 produced a synergistic effect when combined with anti-PD-1 immunotherapy with 70% of the fibrosarcoma and 80% of colorectal adenocarcinoma mice noted to be tumor free [119]. This regimen, however, was shown to have no effect on the melanoma model. Interestingly, treating mice with anti-LAG-3/anti-PD-1 combined therapy is proposed to be less toxic given that LAG-3 and PD-1 co-expression is largely limited to tumor infiltrating lymphocytes.

IMP321 is a clinical grade LAG-3-Ig recombinant fusion protein that antagonizes normal LAG-3 functioning [120]. In 2009 the results of the first Phase I study involving IMP321 alone was released. Patients with advanced renal cell carcinoma were treated [121]. No significant adverse events occurred. Tumor growth reduction was seen and progression-free survival was better in those patients receiving higher doses (>6 mg). Out of eight patients treated with high dose IMP321, seven had stable disease at 3 months compared with only three of 11 in the lower dose group. Another Phase I trial of IMP321 and paclitaxel in metastatic breast cancer was conducted [122]. Patients treated with IMP321 were found to have a sustained increase in the number and activation of monocytes and DCs as well as an increase in the percentage of NK and long lived cytotoxic effector memory CD8 T cells, which correlates well with the preclinical data. Additionally 90% of patients had some clinical benefit, with only three of 12 patients progressing by 6 months. Objective tumor response rate was 50% compared with the historic control group of 25% [122]. IMP321 is well tolerated as well. There were several other Phase I trials combining IMP321 with other agents. In 18 patients with advanced pancreatic adenocarcinoma, IMP321 was combined with conventional gemcitabine and had a good safety profile [123]. Its clinical benefit was hard to evaluate likely due to suboptimal dosing of gemcitabine. IMP321 was combined with an anticancer vaccine MART-1 peptide and used in 12 patients with advanced melanoma [124]. Six patients received MART-1 alone and six in combination with IMP321. One patient experienced a partial response in the IMP321 group and none in the MART-1 alone group.

BTLA

BTLA (CD272) is a transmembrane protein that is expressed on Th1 cells as well as B cells and DCs [125–127]. BTLA, via interaction with HVEM (herpes virus entry mediator), inhibits cancer specific CD8 T cells [127]. HVEM is expressed in hematopoietic cells, including B and T cells, as well as in nonhematopoietic cells (parenchymal cells) [126]. HVEM is also expressed in melanoma cells and variety of solid tumors [126]. Hodgkin's lymphoma, B-cell non-Hodgkin's lymphoma and some T-cell non-Hodgkin's lymphomas use BTLA for immune evasion [127]. BTLA and HVEM are highly expressed in B-cell chronic lymphocytic leukemia suggesting their role in pathogenesis [126,128]. BTLA–HVEM is implicated in Vγ9Vδ2 T-cell proliferation, differentiation and has a critical role in their control of lymphogenesis [129]. T-cell responses against minor histocompatibility antigens on malignant cells play an important role for cure in hematological malignancies after allogeneic stem cell transplant via graft versus tumor effect. BTLA suppresses minor histocompatibility antigen-specific CD8 T cells after allogeneic stem cell transplant thus providing a rationale for its clinical utility in post transplantation therapies [130]. High BTLA expression is associated with shorter survival in gastric cancer [131]. BTLA expression is upregulated on cytotoxic CD8 T cells in peripheral blood of patients with hepatocellular carcinoma and correlates with disease progression and increased expression is associated with high recurrence rates [132]. Downregulation of BTLA *in vivo* can be attained by adding CpG oligonucleotides to vaccine formulation, which leads to increased resistance to BTLA/HVEM inhibition [133]. Because of the role of BTLA/HVEM in hematological as well as solid tumors, BTLA inhibition also represents a target for cancer immunotherapy.

IDO synthase

Tryptophan plays an important role in peripheral immune tolerance through its rate limiting tryptophan degradation along the kynurenine pathway, including IDO1 and tryptophan-2,3-dioxygenase (TDO). Shortage of tryptophan leads to cell cycle arrest, decreased proliferation through inactivation of mTOR pathway, while tryptophan metabolites can cause T-cell apoptosis, and induce differentiation of Tregs. Tumors can evade the immune system by hijacking tryptophan catabolizing enzymes IDO1 and TDO [134]. IDO1 protein is expressed in mature DCs in lymphoid tissues, some epithelial cells of female genital tract, placental and pulmonary endothelial cells [135]. IDO1 positive cells are scattered in human and tumoral lymphoid tissues particularly in cervical, colorectal and gastric carcinomas. It is highly present in vascular cells

Table 1. Summary of clinical trials with agents targeting immunomodulatory pathways beyond CTLA-4 and PD-1/PD-L1.

Agent	Phase	Design	Enrolment	Setting	Completion date	NCT
CD27						
Varlilumab (CDX-1127)	I	Dose escalation (0.1–10 mg/kg)	170	B-cell malignancies and solid tumors	December 2015	NCT01460134
	I	Stereotactic body radiation therapy randomized to before, after or with immunotherapy	21	Castrate resistant prostate cancer	March 2017	NCT02284971
	Ib	Two dose levels in combination with vaccine ONT-10	42	Breast and ovarian cancer	May 2016	NCT02270372
	I/II	Dose escalation of varlilumab with nivolumab	190	Advanced solid tumors	December 2017	NCT02335918
	I/II	Dose escalation of varlilumab with sunitinib	58	Metastatic clear cell carcinoma	March 2019	NCT02386111
	I/II	Dose escalation of varlilumab with ipilimumab	100	Unresectable stage III or IV melanoma	December 2020	NCT02413827
OX40 & OX40L						
MEDI6469	Ib	Dose escalation study of drug given at different time intervals before surgery	55	Advanced head and neck squamous cancer	October 2019	NCT02274155
	I/II	In combination with radiation therapy and escalating doses of cyclophosphamide	37	Metastatic prostate cancer	October 2016	NCT01303705
	Ib/II	Alone or in combination with tremelimumab, MEDI4736 or rituximab	212	Advanced solid tumors or diffuse large B-cell lymphoma	October 2017	NCT02205333
MEDI0562	I	First in human	50	Selected solid tumors	November 2019	NCT02318394
MEDI6383	I	Alone or in combination with MEDI4736	212	Advanced solid tumors	April 2019	NCT02221960
Anti OX40 mab	I/II	Stereotactic body radiation therapy to metastatic lesions with anti OX49 antibody	40	Breast cancer patients with liver or lung metastasis	February 2023	NCT01862900
Anti OX40 mab 9B12	I	Dose escalation study	30	Advanced cancer	January 2015	NCT01644968
CD40 & CD40L						
Dacetuzumab (SGN-40)	I	Dose escalation	50	Relapsed or refractory non-Hodgkin's lymphoma	March 2007	NCT00103779
	I	Dose escalation	44	Recurrent or refractory multiple myeloma	November 2007	NCT00079716
	I	Dose escalation in combination with lenalidomide and dexamethasone	36	Relapsed multiple myeloma	February 2010	NCT00525447
	Ib	In combination with bortezomib	18	Multiple myeloma	April 2010	NCT00664898

Data taken from [86].

Table 1. Summary of clinical trials with agents targeting immunomodulatory pathways beyond CTLA-4 and PD-1/PD-L1 (cont.).

Agent	Phase	Design	Enrolment	Setting	Completion date	NCT
CD40 & CD40L (cont.)						
	Ib	In combination with rituximab	22	Follicular and marginal zone lymphoma	November 2010	NCT00556699
	Ib	In combination with rituximab and gemcitabine	33	Diffuse large B-cell lymphoma	February 2010	NCT00655837
	II	Monotherapy	46	Relapsed diffuse large B-cell lymphoma	January 2009	NCT00435916
	I/II	Dose escalation	12	Chronic lymphocytic leukemia	October 2006	NCT00283101
CP-870,893	I	Dose escalation in combination with tremelimumab	32	Metastatic malignant melanoma	October 2014	NCT01103635
	I	Dose escalation study in combination with gemcitabine	22	Unresectable pancreatic cancer	January 2011	NCT00711191
	I	Dose escalation	29	Advanced solid tumors	February 2006	NCT02225002
	I	Dose escalation in combination with carboplatin and paclitaxel	34	Metastatic solid tumors	July 2009	NCT00607048
Lucatumumab (HCD122)	I	Dose escalation	28	Multiple myeloma	May 2009	NCT00231166
	1b	In combination with bendamustine	?	Follicular lymphoma	May 2012	NCT01275209
	Ia/II	Dose escalation	111	Lymphomas	February 2013	NCT00670592
SEA-CD40	I	Dose escalation	144	Advanced solid tumors	March 2019	NCT02376699
APX005M	I	Dose escalation	32	Advanced solid tumors	December 2017	NCT02482168
Recombinant CD40L	I	Dose escalation in combination with recombinant FLT3 ligand	?	Metastatic melanoma or kidney cancer	Completed	NCT00020540
Chi Lob 7/4	I	Dose escalation	29	Advanced cancer	October 2014	NCT01561911
ADC–1013	I	Dose escalation	40	Advanced solid tumors	October 2017	NCT02379741
AdCD40L	I/IIa	Alone or in combination with low dose cyclophosphamide with or without radiation	30	Advanced cancer	December 2017	NCT01455259
RO7009789	Ib	Dose escalation in combination with MPDL3280A	160	Advanced solid tumors	December 2017	NCT02304393

Data taken from [86].

Table 1. Summary of clinical trials with agents targeting immunomodulatory pathways beyond CTLA-4 and PD-1/PD-L1 (cont.).

Agent	Phase	Design	Enrolment	Setting	Completion date	NCT
CD137 & CD137L						
Urelumab (BMS663513)	I	Dose escalation	122	Advanced cancers	March 2019	NCT01471210
	Ib	In combination with cetuximab	104	Advanced colorectal carcinoma or head and neck squamous cell carcinoma	January 2017	NCT02110082
	Ib	Dose escalation in combination with rituximab	74	B-cell non-Hodgkin's lymphoma	December 2018	NCT01775631
	I/II	Dose escalation in combination with nivolumab	200	Advanced cancers	December 2018	NCT02253992
	II	In combination with rituximab	24	Advanced chronic lymphocytic leukemia	July 2020	NCT02420938
PF-05082566	I	Dose escalation and in combination with rituximab in B-cell lymphomas	126	Advanced solid tumors or B-cell non-Hodgkin's lymphoma	December 2017	NCT01307267
	Ib	Dose escalation in combination with mogamulizumab	70	Advanced cancers	June 2018	NCT02444793
	Ib	Dose escalation in combination with pembrolizumab	45	Advanced solid tumors	May 2017	NCT02179918
GITR						
TRX518	I	Dose escalation	40	Advanced solid tumors	December 2015	NCT01239134
LAG-3						
IMP321	I	Dose escalation	24	Advanced renal cell carcinoma	October 2008	NCT00351949
	I	Dose escalation in combination with paclitaxel	33	Metastatic breast cancer	January 2010	NCT00349934
	I	Dose escalation in combination with gemcitabine	18	Advanced pancreatic cancer	September 2012	NCT00732082
BMS-986016	I	Dose escalation in combination with nivolumab	198	Advanced solid tumors	May 2018	NCT01968109
	I	Dose escalation	88	Chronic lymphocytic leukemia, lymphomas and multiple myeloma	June 2018	NCT02061761
LAG525	I/II	Dose escalation in combination with PDR001	240	Advanced solid tumors	November 2017	NCT02460224

Data taken from [86].

in renal cell cancer [135]. Expression of IDO1 is highest in endometrial and cervical cancers followed by kidney and lung [135]. IDO1 expression is associated with aggressive phenotype, poor prognosis, shorter survival and increased Tregs [136]. IDO1 inhibitors were shown to exhibit antitumor effect in mouse models alone as well as have synergistic effects with a variety of chemotherapeutic agents in preclinical models [137–140]. Indoximod is an IDO inhibitor that is being studied in a Phase II trial with taxane chemotherapy in metastatic breast cancer (NCT 01792050 [86]). IDO inhibitors can provide a synergistic effect when administered with vaccines and immunotherapy; IDO induction in response to inflammation can attenuate antitumor vaccine [139]. This provided the rationale for a randomized Phase II study of Indoximod with Sipuleucel-T (Provenge®) in the treatment of patients with asymptomatic or minimally symptomatic metastatic castration resistant prostate cancer (NCT01560923 [86]). Other studies include a Phase I study of NLG–919, an IDO inhibitor, for patients with advanced solid tumor malignancies and a Phase I/II trial of the indoximod in combination with ipilimumab for the treatment of unresectable advanced stage melanoma [141,142].

Conclusion & future perspective

Our understanding of immune dysfunction in cancer continues to develop. Growth and progression of cancer are made possible by the ability of the malignant cells to manipulate immune checkpoint pathways that prevent immune overstimulation. Significant progress has been made in the field with mAb against CTLA-4 and now PD-1 in clinical use. This gives credence to the efforts aimed at developing agents targeting other immune modulatory pathways. The success PD-1 inhibitors may very well translate to PD-L1 inhibitors being successful in the clinic. PD-L1 is widely expressed in a variety of tumors and PD-L1 inhibitors have shown impressive, albeit preliminary, results in areas of unmet need such as urothelial bladder cancer which has been resistant to conventional chemotherapy [34,50]. Other B7 family members are emerging as clinical target in preclinical models such as B7x, HHLA2 and B7-H3. Other immunoglobulin superfamily members such as Tim-3 and VISTA also show promise in preclinical models. LAG-3 targeting molecules have also reached clinical trials with good safety profile and evidence of antitumor efficacy.

TNFR superfamily member also hold promise in cancer immunotherapy. Preclinical and clinical data support targeting CD27 where a durable response was noted as well [81,82]. mAb targeting CD40L have reached clinical trials although the clinical experience in the CD40/CD40L pathway illustrates some of the caution needed with immune disinhibition, particularly in unwanted side effects such as cytokine release syndrome [88,92,93]. OX40, CD137 and GITR antibodies have also reached clinical trials providing further evidence that TNFR family members are viable targets for cancer immunotherapy [101–104,112]. Table 1 is the summary of clinical trials with agents targeting immunomodulatory pathways beyond CTLA-4 and PD-1/PD-L1.

The redundancy in inhibitory immune checkpoint molecules sheds light on the complexity of immune regulation. It also allows for the targeting of these molecules in succession or in combination. A new pathway may be targeted after the efficacy of an earlier immunotherapy is exhausted as evidenced by the success of PD-1 targeting mAbs in ipilimumab-refractory tumors indicating that tumors may recruit additional pathways when one is blocked, or several pathways may be in fact be recruited simultaneously by tumor cells [24,25,31]. Efficacy of ipilimumab in combination with nivolumab also illustrates that tumors may recruit more than one inhibitory pathway at the same time [143].

Another interesting dilemma that emerges as cancer immunotherapy gains momentum is integrating these novel agents with current regimens that mainly consist of conventional cytotoxic chemotherapy. On one hand, conventional wisdom may lead us to believe that chemotherapy may be synergistic. The immunosuppressive effect of chemotherapy however can have unpredictable effects on the immune system. Cyclophosphamide has been used as a Treg depleting agents in preclinical models with some success [144,145]. Combination regimens have also reached clinical trials [34,122,123]. Combination of immune checkpoint inhibitors have also been studied with traditional immunotherapies or targeted therapies such as rituximab and cetuximab where they show efficacy and tolerability [105,106,146]. With evidence pointing to better outcomes in second line setting or even first line setting of immunotherapies, cancer immunotherapy will play a vital role in the future of oncology [24,31,147].

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Executive summary

Cancer immunotherapy targets in clinical trials

- PD L-1 blockade can restore cytotoxic T-lymphocyte antitumor activity.
- CD27 is a hematologic malignancy marker that has been targeted in Phase I trials.
- Lag-3 is a co-inhibitory molecule and blocking antibodies have reached clinical trials.
- CD40, CD137 and GITR agonism may play a role in cancer immunotherapy and have reached Phase I and Phase II trials.
- Indoleamine 2,3-dioxygenase is not a cell-surface protein and inhibitors have reached clinical trials.

Emerging targets for cancer immunotherapy still in preclinical study

- B7x, HHLA 2, B7-H3 are B7 family members that have inhibitory role and are expressed by tumor cells.
- Tim-3, VISTA and BTLA blockade are emerging targets in preclinical models.
- Agonism of stimulatory molecules OX40 and OX40L may also play a role in cancer immunotherapy.

Conclusion

- T-cell disinhibition is now a clinically effective approach.
- Targeting of several pathways may be done in succession or in concert.
- Immunotherapy can be more effective than chemotherapy especially in the second-line setting.

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